Profiling of vaginal *Candida* isolates from South African women: Exploring potential alternative therapeutic strategies

Presented by

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Co-Supervisor: Professor Nathlee Abbai

PREFACE

Candida albicans (*C. albicans*) remains one of the most clinically significant fungal pathogens affecting women of reproductive age, contributing to both acute vulvovaginal candidiasis (VVC) and recurrent vulvovaginal candidiasis (RVVC). The rising burden of antifungal resistance among *C. albicans* isolates, coupled with strain diversity, biofilm formation, and host-related susceptibility factors, continues to challenge effective management of this infection. In South Africa, unique demographic and clinical determinants, including high human immunodeficiency virus (HIV) prevalence, shape women's vulnerability to RVVC. The limited availability of local genotypic, phenotypic, and molecular data has restricted efforts to fully characterise *C. albicans* within this population.

The research presented in this thesis was therefore conducted to address these gaps by examining *C. albicans* isolates from South African women through a multifaceted investigative approach. This study explored the organism's genotypic diversity using ABC genotyping, assessed antifungal susceptibility patterns with an emphasis on fluconazole resistance, analysed molecular resistance mechanisms through *ERG11* gene expression and phylogenetic profiling, and evaluated the antifungal and antibiofilm potential of natural therapeutics, including plant-derived nanoemulsions and *Lactobacillus* cell-free supernatants (CFSs). Together, these complementary methodologies offer a comprehensive and integrated understanding of *C. albicans* behaviour, resistance evolution, and potential avenues for alternative treatment within this context.

The experimental work described in this thesis was conducted at the School of Clinical Medicine Research Laboratory and the Department of Medical Microbiology, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal (UKZN), Durban, South Africa. All work was carried out under the supervision of Doctor Refilwe Phemelo Molatlhegi and the co-supervision of Professor Nathlee Abbai. This study contributes foundational knowledge to the growing field of *Candida* research in South Africa and provides critical insights to guide future surveillance, diagnostic development, and the exploration of novel therapeutic strategies.

PLAGIARISM DECLARATION

I **Caitlin Ramnarain** declare that:

(i) The research reported in this dissertation, except where otherwise indicated, is my original work.

(ii) This dissertation has not been submitted for any degree or examination at any other university.

(iii) This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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Signed: [redacted] Date: 27 November 2025

PERMISSION TO SUBMIT

As the candidate's supervisors, we confirm that we have read the thesis in full and approve its submission for examination.

Supervisor: Doctor Refilwe Phemelo Molatlhegi
Date: 27 November 2025

Co-supervisor: Professor Nathlee Abbai
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RESEARCH OUTPUTS

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2. **Caitlin Ramnarain,** Anmol Gokul, Veron Ramsuran, Refilwe Phemelo Molatlhegi and Nathlee Abbai. *ERG11* overexpression and genetic divergence drive fluconazole resistance in clinical *Candida albicans*: A droplet digital polymerase chain reaction and phylogenetic analysis. Submitted to *International Journal of Microbiology* (Under Review). Manuscript ID: 3361235.

3. **Caitlin Ramnarain,** Nathlee Abbai and Refilwe Phemelo Molatlhegi. Antibiofilm and antifungal activity of natural therapeutics against *Candida albicans*: A focus on plant nanoemulsions and *Lactobacillus* supernatants. Submitted to *International Journal of Microbiology* (Under Review). Manuscript ID: 2957550.

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1. Kehinde Charles Mofolorunsho, **Caitlin Ramnarain,** Nonkululeko Mabaso, Nikita Nundlall and Nathlee Abbai. *Chlamydia trachomatis* genotypes among men who have sex with men in Durban, South Africa. *Journal of Medical Laboratory Science & Technology of South Africa 2024;* 6:31-41. https://doi.org/10.36303/JMLSTSA.170

PRESENTATIONS

1. **Caitlin Ramnarain,** Gloria Sukali, Ntombizethu Msomi, Nonkululeko Mabaso, Refilwe Phemelo Molatlhegi and Nathlee Abbai. Correlation between genotypes and antifungal susceptibility profiles of *Candida* isolates from pregnant and non-pregnant women in South Africa. Poster presentation at the College of Health Sciences Annual Research Symposium, University of KwaZulu-Natal, Durban, South Africa (25th August 2025).

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3. **Caitlin Ramnarain.** Profiling of vaginal *Candida* isolates from South African women: Exploring potential alternative therapeutic strategies. Oral presentation at the Department of Medical Microbiology's Annual Research Update, University of KwaZulu-Natal, Durban, South Africa (3rd June 2023).

DEDICATION

I dedicate this thesis first and foremost to my Heavenly Father and my Lord and Saviour, Jesus Christ. Thank You, Father, for Your grace, strength, divine guidance and unending love throughout every season of this journey. Your Word sustained me daily:

"For I know the plans I have for you, declares the Lord, plans to prosper you and not to harm you, plans to give you hope and a future." - Jeremiah 29:11

and

"I can do all things through Christ who strengthens me." - Philippians 4:13

Your promises have been the anchor of my soul, and every chapter of this thesis is a testament to Your faithfulness.

This thesis is also dedicated, with all my heart, to my beloved Dad, Rakesh Ramnarain, who left this world too soon. Dad, your memory lives on in every part of my life. Though you are no longer here with me, your love, wisdom, strength, and the dreams you carried for me continue to guide my steps. I carry you with me always. This achievement is as much yours as it is mine.

To my Mum, Daphne Ramnarain, my pillar of strength, thank you for your unconditional love, your countless sacrifices, your prayers, and your support throughout every stage of my academic and personal journey.

To my incredible husband, Nischkar Nirmal, whose unwavering love carried me through every late night and every challenge. Thank you for being the love of my life, my best friend, my greatest comfort, and my number-one supporter. Your patience, understanding, and steadfast pride in everything I do have been my constant source of strength.

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Lastly, to all my family, my spiritual family from Fragrant Oil Fellowship, and my friends who cheered me on, prayed for me, supported me, and believed in me, thank you from the depths of my heart. This journey would not have been possible without your presence, love, and encouragement.

To everyone who stood by me, this thesis reflects your love, sacrifice, and belief in me. I thank God for each one of you.

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I would like to express my deepest gratitude to my supervisor, **Dr. Refilwe Phemelo Molatlhegi,** from the Department of Medical Microbiology, and my co-supervisor, **Prof. Nathlee Abbai,** from the Department of Clinical Medicine, for their invaluable guidance, mentorship, and unwavering support throughout this PhD journey. Their scientific insight, dedication, and encouragement have been instrumental in shaping both this research and my development as a scientist. I am immensely grateful for their expertise, thoughtful feedback, and steadfast commitment, all of which strengthened this project in every aspect. Their combined leadership has truly been a tremendous blessing.

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I would also like to acknowledge **Dr. Ntombizethu Msomi,** who was responsible for the collection of the *Candida* isolates used in this project. Your contribution formed the foundation upon which much of this work was built, and your efforts are sincerely appreciated.

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ABC	Adenosine triphosphate-binding cassette
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
ART	Antiretroviral therapy
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
bp	base pair
BREC	Biomedical Research Ethics Committee
CD4+	Cluster of differentiation 4-positive
CFS	Cell-free supernatant
CLSI	Clinical and Laboratory Standards Institute
Ct	Cycle threshold
ddPCR	droplet digital polymerase chain reaction
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
HIV	Human immunodeficiency virus
ICU	Intensive care unit
Kor-GLASS	Korea Global Antimicrobial Resistance Surveillance System
LOH	Loss of heterozygosity
MCL	Maximum Composite Likelihood
MIC	Minimum inhibitory concentration
MIF	Minimum inhibitory factor
ml	millilitre
MLST	Multilocus sequence typing
mm	Millimetre
MRS	Man, Rogosa, and Sharpe
MTP	Microtiter plate
NAC	Non-albicans *Candida*
NIC	Neonatal invasive candidiasis
NICU	Neonatal intensive care unit
NJ	Neighbor-Joining
nm	nanometre
OD	Optical density

OPC	Oropharyngeal candidiasis
OEC	Oesophageal candidiasis
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
cDNA	Complementary deoxyribonucleic acid
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
rpm	revolutions per minute
RVVC	Recurrent vulvovaginal candidiasis
SD	Standard deviation
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
SDD	Susceptible-dose-dependent
SENTRY	Secure Electronic Network for Travelers Rapid Inspection
UKZN	University of KwaZulu-Natal
VVC	Vulvovaginal candidiasis

LIST OF SYMBOLS

≈	Approximately
cells/µl	cells per microliter
copies/µl	copies per microliter
°C	Degrees Celsius
=	Equals
△△Ct	Fold change
G	Grams
>	Greater than
≥	Greater than or equal to
<	Less than
≤	Less than or equal to
Log_2(△△Ct)	Log_2 fold change
µg/ml	micrograms per millilitre
µl	Microlitre
µm	Micrometre
µM	Micromolar
-	Minus
×	Multiplication
ng/µl	nanograms per microliter
△Ct	Normalized expression
%	Percentage
+	Plus
±	Plus-minus
pH	potential of hydrogen
P	Probability
× *g*	Relative centrifugal force
n	Sample size
U/µl	units per microliter
v/v	volume/volume

LIST OF APPENDICES

ABSTRACT

Introduction: *Candida albicans* remains the leading cause of vulvovaginal candidiasis (VVC), posing a persistent threat to women's reproductive health globally. Increasing resistance to azole antifungals, driven by strain diversity, biofilm formation, and molecular mechanisms such as *ERG11* overexpression, has complicated treatment outcomes and contributed to recurrent infections. Despite extensive global research, there is limited data from South Africa regarding genotype distribution, resistance patterns, and the role of alternative therapeutics.

Methods: A cross-sectional study was conducted using stored *Candida* isolates obtained from pregnant and non-pregnant women attending Victoria Mxenge Hospital in Durban, South Africa. Isolates were confirmed as *C. albicans* using germ-tube testing and molecular identification. Antifungal susceptibility was assessed via broth microdilution to determine fluconazole minimum inhibitory concentrations (MICs). ABC genotyping of the *25S ribosomal deoxyribonucleic acid (rDNA)* region classified isolates into genotypes A, B, and C. Molecular analysis involved ribonucleic acid (RNA) extraction, complementary deoxyribonucleic acid (cDNA) synthesis, and droplet digital PCR (ddPCR) to quantify *ERG11* expression. Phylogenetic analysis assessed genetic relationships. Additionally, nanoemulsions derived from *Azadirachta indica*, *Moringa oleifera*, and *Ocimum tenuiflorum*, along with *Lactobacillus* cell-free supernatants (CFS), were evaluated for antifungal and antibiofilm activity using MIC and microtiter plate (MTP) assays.

Results: All 72 isolates were identified as *C. albicans*, with genotype A predominating (62.5%), followed by genotype B (26.4%) and genotype C (11.1%). Fluconazole resistance was observed in 13.9% of isolates, with MICs ranging from 8 µg/mL to >256 µg/mL. Resistance was strongly associated with genotypes A and C, while genotype B remained fully susceptible. Significant *ERG11* overexpression (1.31-3.73-fold) was detected in most resistant isolates, although one isolate demonstrated resistance independent of *ERG11* upregulation. Phylogenetic analysis revealed clustering of resistant strains. While fluconazole was ineffective against resistant biofilms, plant nanoemulsions at 1000 µM completely inhibited biofilm formation, and *Lactobacillus* CFS significantly reduced early biofilm development, though efficacy varied by strain.

Conclusion: These findings highlight emerging resistance trends and support the potential of alternative therapeutics in managing drug-resistant and recurrent VVC (RVVC) in South Africa.

CHAPTER 1

1.1 Thesis Overview

1.1.1 Thesis structure

This thesis follows the thesis-by-manuscript format stipulated by the College of Health Sciences at the University of KwaZulu-Natal. Each manuscript is presented in accordance with the specific formatting and submission requirements of its respective journal. The thesis is guided by the following chapters:

Chapter 1: This chapter provides a review of the literature on *C. albicans*, consisting of an introduction to the study, a description of the problem statement, research questions, rationale, aim, and objectives of the study. The literature review titled **"The rising threat of antifungal resistance: A comprehensive review of *Candida* infections and vulnerable populations."** is presented in the format of a manuscript that was submitted to *MicrobiologyOpen* (Under Review). Manuscript ID: 3028254.

Chapter 2: This chapter is entitled: **"Correlation between genotypes and antifungal susceptibility profiles of *Candida* isolates from pregnant and non-pregnant women in South Africa."** This paper characterises the genetic diversity of *C. albicans* isolates using ABC genotyping and correlates these genotypes with antifungal susceptibility profiles among pregnant and non-pregnant South African women. This manuscript was submitted to *Journal of Medical Laboratory Science & Technology of South Africa 2025;* 7(2):6-15 and was published (Appendix C) on the 24th of November 2025. https://doi.org/10.36303/JMLSTSA.282

Chapter 3: This chapter is entitled: **"*ERG11* overexpression and genetic divergence drive fluconazole resistance in clinical *Candida albicans*: A droplet digital polymerase chain reaction and phylogenetic analysis."** This paper investigates the molecular basis of fluconazole resistance by quantifying *ERG11* gene expression using droplet digital polymerase chain reaction and analysing the phylogenetic relationships of resistant and susceptible *C. albicans* isolates. This manuscript was submitted to *International Journal of Microbiology* (Under Review). Manuscript ID: 3361235.

Chapter 4: This chapter is entitled: **"Antibiofilm and antifungal activity of natural therapeutics against *Candida albicans*: A focus on plant nanoemulsions and *Lactobacillus* supernatants."** This paper evaluates the antifungal and antibiofilm efficacy of plant-based nanoemulsions and *Lactobacillus*-derived supernatants against clinical *C. albicans* isolates, highlighting their potential as complementary therapeutic agents. This manuscript was submitted to *International Journal of Microbiology* (Under Review). Manuscript ID: 2957550.

Chapter 5: This final chapter presents an integrated synthesis of the study, contextualizing its key findings within existing literature. It further outlines the study's strengths, and limitations, and

concludes with the overarching insights derived from the project alongside recommendations for future research.

1.1.2 Study design and Methodology

Ethics approval for this research was obtained from the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (UKZN) (BREC/00005995/2023). This study forms part of a broader investigation (BREC/00003674/2021), conducted within the Clinical Medicine Research Laboratory at the Nelson R. Mandela School of Medicine, UKZN. The research utilised stored *C. albicans* isolates (n = 72) obtained from a previous cross-sectional study conducted among pregnant and non-pregnant women attending public healthcare facilities in Durban, South Africa. The parent study recruited participants who provided demographic, clinical, and behavioural data, as well as self-collected vaginal swabs for microbiological analysis.

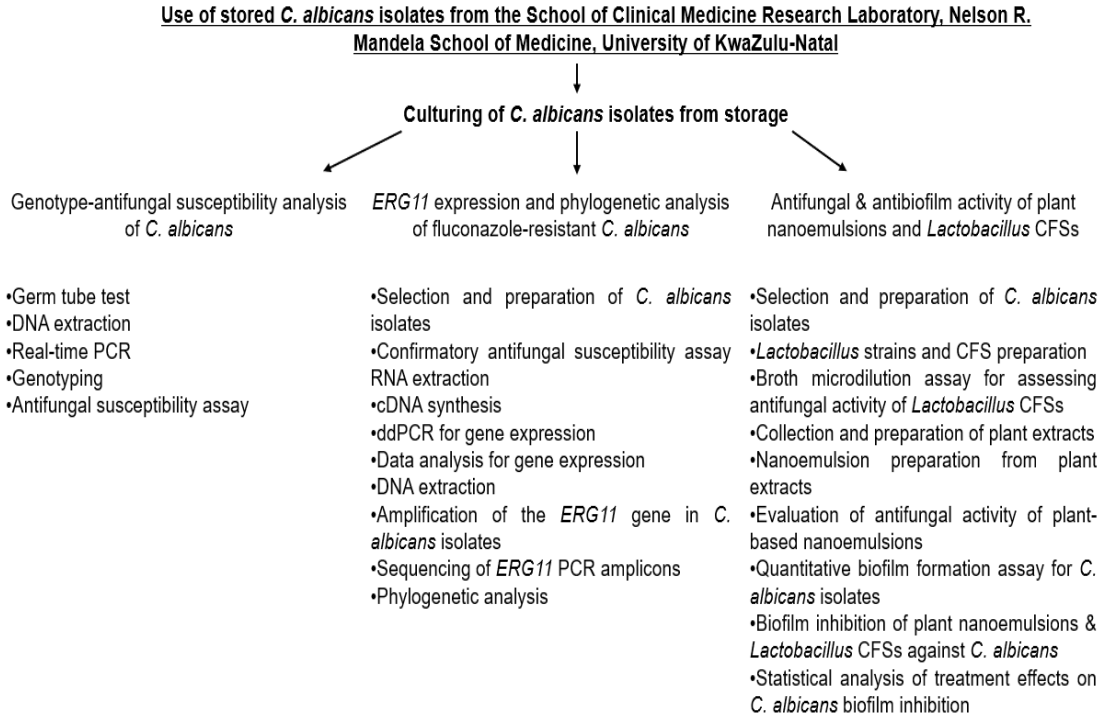
A comprehensive laboratory-based approach was employed to investigate phenotypic antifungal susceptibility, strain diversity, molecular mechanisms of azole resistance, and the therapeutic potential of natural antifungal agents. The study encompassed several interconnected experimental components. First, stored *C. albicans* isolates were revived, sub-cultured, and confirmed using germ-tube testing and molecular identification assays. Antifungal susceptibility testing was performed using the broth microdilution method to determine minimum inhibitory concentrations (MICs) for fluconazole and related azole agents.

Genotypic characterization of the isolates was conducted using the ABC genotyping method, which involves amplification of the *25S rDNA* region. This approach allows for classification of isolates into genotypes A, B, C, or D, enabling exploration of potential associations with antifungal susceptibility patterns. Molecular evaluation of fluconazole resistance mechanisms involved RNA extraction, cDNA synthesis, and quantification of *ERG11* gene expression using ddPCR assays. Phylogenetic analysis was conducted to assess evolutionary relationships between resistant and susceptible isolates.

To explore alternative therapeutic options, plant-derived nanoemulsions formulated from *A. indica*, *M. oleifera*, and *O. tenuiflorum*, extracts were synthesised and characterised before being tested against planktonic and biofilm-forming *C. albicans*. *Lactobacillus* CFSs were also prepared and evaluated for their inhibitory effects on fungal growth and biofilm development. Antifungal and antibiofilm activity were assessed using MIC and MTP assays.

All experimental work was conducted at the Clinical Medicine Research Laboratory at the Nelson R. Mandela School of Medicine and the Department of Medical Microbiology, UKZN. Data generated from the various experimental procedures contributed to a comprehensive analysis of the epidemiological, genetic, phenotypic, and molecular characteristics of *C. albicans*, as well as an

evaluation of natural therapeutic strategies with potential clinical applicability. The flow diagram presented below summarises the overall methodology employed in this thesis.



1.2 Introduction

Fungal infections remain a significant and often under-recognised public health problem globally, affecting millions of individuals each year and contributing substantially to morbidity, reduced quality of life, and increased healthcare expenditure. Among these infections, vulvovaginal candidiasis (VVC) represents one of the most common conditions affecting women, particularly those of reproductive age, with *Candida albicans* (*C. albicans*) identified as the predominant causative agent in both uncomplicated and recurrent cases (Gonçalves *et al.*, 2016; Denning *et al.*, 2018). Although VVC is typically considered a benign mucosal infection, its impact is far-reaching. Up to 75% of women will experience at least one episode during their lifetime, and approximately 138 million women are affected by recurrent VVC (RVVC) annually, highlighting its immense global burden (Denning *et al.*, 2018). Women suffering from RVVC frequently report substantial physical discomfort, emotional distress, impairment in sexual and reproductive well-being, and disruption of daily activities, underscoring the multidimensional burden of this condition (Sobel, 2016). Recent reports even suggest an upward trend in RVVC incidence due to factors including widespread antibiotic use, contraceptive hormonal modulation, and the rising prevalence of antifungal-resistant strains (de Cássia Orlandi Sardi *et al.*, 2021).

The epidemiology of *Candida* infections has undergone considerable shifts over the last two decades, with an increasing number of infections caused by non-*albicans Candida* (NAC) species such as *Candida glabrata* (*C. glabrata*), *Candida tropicalis* (*C. tropicalis*), *Candida parapsilosis* (*C. parapsilosis*), and the emerging pathogen *Candida auris* (*C. auris*) (Pappas *et al.*, 2018). NAC species often possess intrinsic or inducible resistance to azoles, which complicates therapeutic management and contributes to treatment failure (Arendrup and Patterson, 2017). In sub-Saharan Africa, including South Africa, NAC species are isolated with greater frequency in women with RVVC or persistent VVC, suggesting complex ecological and host-driven influences on species distribution (Donders, Bellen and Mendling, 2010; Sobel, 2024). These shifts may be driven by alterations in host immunity, human immunodeficiency virus (HIV) prevalence, widespread exposure to antifungals, environmental pressures, and microbiome dysbiosis (Zaongo *et al.*, 2023). Concurrently, the global rise in antifungal resistance has been recognised as an urgent health threat, prompting the World Health Organization (WHO) to prioritise *C. albicans*, *C. glabrata,* and *C. auris* as fungal pathogens requiring immediate research attention (Organization, 2022).

Antifungal therapy for *Candida* infections is dominated by four major drug classes: azoles, polyenes, echinocandins, and flucytosine (Cowen *et al.*, 2015). Of these, fluconazole remains the first-line treatment for VVC due to its favourable pharmacokinetic properties, oral availability, affordability, and widespread familiarity among clinicians (Pappas *et al.*, 2018). However, increasing fluconazole resistance has emerged in both *C. albicans* and NAC species, driven by repeated or prophylactic azole exposure, inappropriate antifungal prescribing, and clonal expansion of resistant strains (Perlin,

Rautemaa-Richardson and Alastruey-Izquierdo, 2017). South African surveillance studies have similarly reported rising azole non-susceptibility and significant changes in species distribution, emphasizing the need for robust local epidemiological monitoring (Naicker *et al.*, 2016). Resistance not only complicates treatment but also poses a risk of recurrent episodes, persistent colonisation, and progression to systemic disease, especially in vulnerable immunocompromised populations.

At the molecular level, azole resistance in *C. albicans* results from intricate adaptive mechanisms. Central to this is the *ERG11* gene, which encodes lanosterol 14α-demethylase, the primary target of azole drugs. Resistance may arise from overexpression of *ERG11*, point mutations that reduce drug binding, or regulatory pathway disruptions that enhance sterol biosynthesis flux (Nishimoto, Sharma and Rogers, 2019; Lee *et al.*, 2020). Mutations in transcription factors such as *TAC1*, *UPC2,* and *MRR1* further amplify efflux pump expression, contributing to multidrug resistance (Arastehfar *et al.*, 2020b). Structural and biochemical analyses have revealed how specific amino acid substitutions in *ERG11* alter the binding landscape for azole drugs, thereby diminishing their inhibitory effect (Flowers *et al.*, 2015). These adaptations are often accompanied by changes in membrane sterol composition, activation of oxidative stress response pathways, and enhanced chaperone activity, illustrating the multifaceted nature of azole resistance (Cernicka and Subik, 2006; Popp *et al.*, 2017).

Biofilm formation adds another critical dimension to *Candida* pathogenesis and antifungal resistance. Biofilms are structured communities of cells embedded within an extracellular matrix that confer substantial protection against antifungal agents and host immune responses (Nett and Andes, 2020). Biofilm-associated cells exhibit up to 1000-fold higher tolerance to azoles compared to planktonic cells due to matrix-mediated drug sequestration, nutrient gradients, the presence of metabolically dormant persister cells, and upregulated stress-response pathways (Wall *et al.*, 2019; Lohse *et al.*, 2020). In the vaginal environment, biofilms contribute to persistent colonisation and recurrent symptoms, making them an important therapeutic target (de Cássia Orlandi Sardi *et al.*, 2021). This barrier to effective therapy is particularly relevant in women with immunosuppression, HIV infection, pregnancy, diabetes mellitus, or disrupted vaginal microbiota, all of which heighten susceptibility to biofilm-associated infections (Achkar and Fries, 2010).

Genetic diversity among *C. albicans* strains further influences pathogenicity, virulence, and antifungal susceptibility. Molecular typing methods, including multilocus sequence typing and microsatellite typing, have demonstrated substantial intra-species variability (Ge *et al.*, 2010). ABC genotyping, a rapid and cost-effective method based on polymerase chain reaction (PCR) amplification of the *25S ribosomal deoxyribonucleic acid (rDNA)* region, allows classification of *C. albicans* into genotypes A, B, C, and D (Fornari *et al.*, 2016; Gharaghani *et al.*, 2022). Globally, genotype A is the most prevalent and has been associated with increased virulence and antifungal resistance (Al-Groom, Ali and Shaqra,

2024). Differences in genotype distribution across regions may reflect host factors, environmental pressures, microevolution, and antifungal exposure patterns (Gerós-Mesquita *et al.*, 2020).

In addition to genotypic variation, transcriptional and physiological differences among isolates also influence treatment outcomes. Overexpression of *ERG11* is a well-established mechanism of fluconazole resistance, and recent high-resolution studies using quantitative polymerase chain reaction (qPCR) have revealed substantial transcriptional activation of sterol biosynthesis pathways in resistant isolates (Doorley *et al.*, 2023). These insights underscore the complexity of resistance and the need for multi-layered approaches that integrate phenotypic susceptibility data, genotypic classification, and molecular analyses.

Given the limitations of current antifungal drugs, increasing resistance rates, and the challenge posed by biofilm-associated tolerance, there is growing interest in plant-derived natural therapeutics, nanotechnology-based drug delivery systems, and probiotic interventions. Several plant extracts, including *Azadirachta indica* (*A. indica*), *Moringa oleifera* (*M. oleifera*), *Ocimum tenuiflorum* (*O. tenuiflorum*), thymol and eugenol-containing botanicals, possess strong antifungal and antibiofilm properties (Naicker, Govender and Abbai, 2024; Gao *et al.*, 2024). Their incorporation into nanoemulsions can significantly enhance solubility, stability, and mucosal penetration, making them promising therapeutic candidates for mucosal infections such as VVC (Faustino and Pinheiro, 2020). *A. indica*-based nanoformulations have been shown to inhibit adhesion, filamentation, and biofilm maturation in *C. albicans*, with minimal haemolytic toxicity (Khan and Javed, 2021). Similarly, *O. tenuiflorum* and *M. oleifera* extracts exhibit broad-spectrum antimicrobial properties that may contribute to alternative treatment strategies for drug-resistant strains (Naicker, Govender and Abbai, 2024).

In parallel, *Lactobacillus* species play a crucial role in maintaining vaginal homeostasis through the production of lactic acid, competitive exclusion, the secretion of antimicrobial peptides, and the modulation of immune responses (van de Wijgert and Verwijs, 2020). Metabolites derived from *Lactobacillus* species have demonstrated the ability to inhibit *C. albicans* adhesion, suppress hyphal growth, disrupt biofilms, and reduce virulence factors (Poon and Hui, 2023). These findings suggest that probiotic-based therapeutics may offer a complementary or adjunctive strategy in preventing or managing RVVC, particularly in women with microbiome dysbiosis or those at risk of drug-resistant infections.

Taken together, the expanding global burden of VVC, the rise of antifungal resistance, the challenges posed by biofilm-associated infections, the diversity among *C. albicans* genotypes, and the growing interest in natural therapeutics collectively highlight the need for comprehensive research. This thesis integrates epidemiological, genotypic, molecular, and therapeutic perspectives to provide a multifaceted understanding of *C. albicans* infections among South African women. By combining genotypic

surveillance data with antifungal susceptibility profiles, exploring *ERG11* expression and gene divergence, and evaluating the efficacy of plant-based nanoemulsions and *Lactobacillus* supernatants, this work aims to contribute evidence that can inform improved prevention, diagnosis, and treatment strategies for *Candida* infections in resource-limited settings.

1.3 Problem statement

Candida infections continue to pose a significant global health challenge, yet their true burden remains underrecognized, particularly in low- and middle-income regions (Bongomin *et al.*, 2017). VVC and RVVC affect millions of women worldwide each year, leading to substantial discomfort, reduced quality of life, and increased healthcare expenditure (Sobel, 2016; Denning *et al.*, 2018). Despite this high prevalence, the epidemiology, strain diversity, and resistance patterns of *C. albicans* in many settings, including South Africa, are poorly characterised. This is concerning, given that antifungal resistance, particularly to fluconazole, has risen globally over the last decade (Whaley, 2018; Fisher *et al.*, 2022). Increasing azole resistance among both *C. albicans* and NAC species complicates clinical management and contributes to persistent or recurrent infections (Arendrup and Patterson, 2017). South African surveillance studies have similarly documented emerging resistance and shifting species distributions, yet comprehensive local data integrating genotypic profiles with antifungal susceptibility remain limited (Naicker *et al.*, 2016; Chibabhai, 2022).

In addition to increasing drug resistance, the biological complexity of *C. albicans* presents further therapeutic challenges. Biofilm formation significantly reduces antifungal penetration and efficacy, making infections more persistent and challenging to treat (Nett and Andes, 2020; Lohse *et al.*, 2020). Biofilm-associated tolerance contributes to recurrent disease, particularly in women with risk factors such as pregnancy, HIV infection, diabetes, or vaginal microbiome disruption (Achkar and Fries, 2010). At the molecular level, mechanisms such as *ERG11* overexpression, point mutations, and efflux pump activation are well-described contributors to azole resistance globally (Nishimoto, Sharma and Rogers, 2019; Lee *et al.*, 2020). However, the extent to which these mechanisms operate in South African clinical isolates remains poorly understood, and few studies have examined the relationship between genotype, gene expression, and phenotypic resistance in local *C. albicans* strains.

Furthermore, although natural therapeutics, such as plant-based nanoemulsions and *Lactobacillus*-derived metabolites, have demonstrated potent antifungal and antibiofilm properties in local and international studies (Poon and Hui, 2023; Naicker, Govender and Abbai, 2024; Gao *et al.*, 2024). Their effectiveness against South African *C. albicans* isolates has not been adequately explored. Limited research into safe, cost-effective alternative therapies presents a critical gap, especially in resource-limited settings where access to advanced antifungals is restricted.

Overall, despite the high prevalence of VVC and the growing global threat of antifungal resistance, there is a lack of integrated research in South Africa that links strain diversity, antifungal susceptibility patterns, molecular resistance mechanisms, and emerging therapeutic alternatives. Without such evidence, effective management of *Candida* infections remains challenging, particularly for women who are pregnant, immunocompromised, or experiencing recurrent disease. Addressing these gaps is crucial for enhancing diagnosis, informing effective treatment strategies, and guiding future research in the local context.

1.4 Research questions

1.4.1 What are the antifungal susceptibility profiles of *C. albicans* isolates obtained from South African women, and what patterns of azole resistance are evident in this population?

1.4.2 How are *C. albicans* isolates genetically distributed according to ABC genotyping, and do specific genotypes show a greater association with fluconazole resistance?

1.4.3 What molecular mechanisms underlie fluconazole resistance in these isolates, specifically in relation to *ERG11* gene expression levels and phylogenetic clustering?

1.4.4 Do *O. tenuiflorum*, *A. indica,* and *M. oleifera* plant-based nanoemulsions exhibit antifungal and antibiofilm activity against planktonic and biofilm-forming *C. albicans* isolates?

1.4.5 To what extent do *Lactobacillus*-derived cell-free supernatants (CFSs) inhibit the growth and biofilm formation of *C. albicans*, and how do these effects compare to those of plant nanoemulsions?

1.4.6 How can the integration of phenotypic, genotypic, molecular, and therapeutic data contribute to a more comprehensive understanding of recurrent and treatment-resistant *Candida* infections in South African women?

1.5 Rationale

Candida infections, particularly VVC and RVVC, remain a significant yet often underappreciated public health concern, especially among women of reproductive age. Despite their high global prevalence and substantial impact on women's physical, emotional, and reproductive well-being, significant gaps remain in understanding the epidemiology, strain diversity, and resistance patterns of *C. albicans* in many regions, including South Africa (Nyirjesy and Sobel, 2003; Denning *et al.*, 2018). These gaps are concerning because antifungal resistance, especially to fluconazole, the most widely used treatment for VVC, continues to rise globally and locally (Whaley, 2018; Fisher *et al.*, 2022). Without accurate, regionally representative data on susceptibility profiles and the molecular

mechanisms driving resistance, clinical decision-making remains limited, and treatment failures become increasingly common.

There is a particular need for research in South Africa, where unique population dynamics, HIV prevalence, socio-economic challenges, and variations in antifungal prescribing may influence *Candida* epidemiology and resistance patterns (Omrani, 2014; Naicker *et al.*, 2016). Yet, few studies have comprehensively examined the relationship between *C. albicans* genotypes, antifungal susceptibility patterns, and clinical outcomes in South African women. ABC genotyping offers valuable insight into strain diversity and potential associations between genotype and resistance, but local data remain sparse and fragmented (Fornari *et al.*, 2016; Gharaghani *et al.*, 2022). Generating this information is essential for surveillance, early detection of emerging resistant strains, and guiding empiric therapy.

Additionally, the molecular basis of azole resistance, particularly the role of *ERG11* mutations and overexpression, has been widely described globally but is poorly characterised in South African clinical isolates (Nishimoto, Sharma and Rogers, 2019; Lee *et al.*, 2020). Understanding whether local resistant strains exhibit similar or distinct molecular signatures, including patterns of *ERG11* gene expression, sequence variations, and phylogenetic clustering, will provide insight into the evolution of resistance within this population. Such information is necessary to inform diagnostic strategies, support antifungal stewardship, and identify potential molecular targets for future therapies. Without integrating phenotypic, genotypic, and molecular data, the true drivers of resistance in the South African context remain unclear.

At the same time, the growing inadequacy of existing antifungal therapies highlights the urgent need to explore alternative, accessible, and culturally acceptable treatment options. Natural therapeutics, including plant-based compounds such as *A. indica*, *M. oleifera,* and *O. tenuiflorum*, have demonstrated promising antifungal and antibiofilm activity internationally, particularly when formulated as nanoemulsions that enhance stability, solubility, and mucosal penetration (Naicker, Govender and Abbai, 2024; Gao *et al.*, 2024). Likewise, *Lactobacillus*-derived metabolites offer a biologically grounded approach to restoring vaginal microbiota balance, inhibiting *Candida* growth, and disrupting biofilms (van de Wijgert and Verwijs, 2020; Poon and Hui, 2023). Yet, despite their potential relevance for resource-limited settings, these natural therapeutics have not been evaluated against South African *C. albicans* isolates. Locally generated data are essential to determine whether these promising alternatives hold therapeutic value for the women most affected by VVC and RVVC.

This study is therefore justified by the need to address multiple, intersecting gaps in the current understanding of *Candida* infections among South African women. By examining strain distribution and genotype-phenotype relationships, characterising molecular mechanisms of fluconazole resistance, and evaluating the antifungal and antibiofilm activity of natural therapeutics, this thesis provides a comprehensive, multidimensional analysis that reflects both clinical and public health priorities. The

integration of epidemiological, molecular, and therapeutic investigations will generate evidence that is directly applicable to clinical practice, antifungal stewardship, and the development of innovative treatment strategies. Ultimately, this research aims to contribute to improved management and prevention of *Candida* infections in a population disproportionately affected by recurrent, resistant, and biofilm-associated disease.

1.6 Aim of the study

This thesis aimed to generate a comprehensive understanding of the epidemiological, phenotypic, genotypic, and molecular characteristics of *C. albicans* isolates obtained from South African women, while evaluating the potential of natural therapeutic strategies to improve the management of VVC.

1.7 Objectives of the study

1.7.1 To determine the antifungal susceptibility profiles of *C. albicans* isolates and describe local patterns of azole resistance.

1.7.2 To characterise the strain diversity of *C. albicans* using ABC genotyping and assess potential associations between genotype and antifungal resistance.

1.7.3 To investigate molecular mechanisms of fluconazole resistance through quantification of *ERG11* gene expression and phylogenetic analysis of resistant and susceptible isolates.

1.7.4 To evaluate the antifungal and antibiofilm activity of *A. indica*, *M. oleifera,* and *O. tenuiflorum*, plant-based nanoemulsions against planktonic and biofilm-forming *C. albicans* isolates.

1.7.5 To assess the inhibitory effects of *Lactobacillus*-derived CFSs on the growth and biofilm formation of *C. albicans*.

1.7.6 To integrate phenotypic, genotypic, molecular, and therapeutic findings to provide a holistic and locally relevant understanding of the factors contributing to recurrent and treatment-resistant *Candida* infections.

1.8 Literature review

The literature review is presented in the format of a manuscript currently under review in *MicrobiologyOpen*, formatted according to the journal's guidelines for authors. Manuscript ID: 3028254.

The rising threat of antifungal resistance: A comprehensive review of *Candida* infections and vulnerable populations

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Abstract

Candida infections continue to pose a serious global health concern, particularly in immunocompromised individuals, pregnant women, and vulnerable age groups. The increasing emergence of antifungal resistance among both *Candida albicans* and non-albicans *Candida* species complicates treatment outcomes and poses a threat to public health. Resistance mechanisms are multifactorial, involving point mutations in key genes such as *ERG11*, *FKS1*, and *FKS2*, as well as overexpression of efflux pumps like *CDR1*, *CDR2*, and *MDR1*. Biofilm formation and genomic adaptations such as aneuploidy and LOH further support persistent infections and treatment failure. Geographical and species-specific variations in resistance are evident, with *Candida auris*, *Candida glabrata*, and *Candida parapsilosis* emerging as major multidrug-resistant pathogens. Host-related risk factors, including human immunodeficiency virus (HIV) infection, pregnancy, and advanced age, significantly contribute to susceptibility to infection and influence disease severity. Furthermore, racial and ethnic disparities in healthcare access and treatment outcomes have resulted in disproportionate infection burdens among African American, Hispanic, Indigenous, and Southeast Asian populations. Conventional antifungals, although still useful, are facing declining effectiveness due to resistance. Promising alternatives, such as plant-based nanoemulsions, probiotics, peptides, and drug repurposing, are being explored; however, clinical integration remains limited. This review underscores the pressing need for precision antifungal strategies, equitable access to healthcare, and ongoing research into innovative therapeutics. An interdisciplinary, patient-centred approach is vital to address the rising tide of *Candida* resistance and ensure better health outcomes globally.

Keywords: *Candida* | antifungal resistance | probiotics | biofilms | health disparities

Introduction

Candida species are among the most prevalent fungal pathogens affecting humans, with infections ranging from superficial mucosal colonization to invasive systemic diseases. Although *Candida albicans* (*C. albicans*) has long been considered the most dominant and virulent species, global epidemiological surveillance has revealed a substantial shift toward non-*albicans Candida* (NAC) species such as *Candida glabrata* (*C. glabrata*), *Candida parapsilosis* (*C. parapsilosis*), *Candida tropicalis* (*C. tropicalis*), and the multidrug-resistant *Candida auris* (*C. auris*) (1, 2). These species are emerging as major contributors to nosocomial bloodstream infections and vulvovaginal candidiasis (VVC), particularly in immunocompromised individuals, pregnant women, and neonates. Surveillance programs, such as the Secure Electronic Network for Travelers Rapid Inspection (SENTRY) and the Korea Global Antimicrobial Resistance Surveillance System (Kor-GLASS), have highlighted geographical differences in species prevalence and antifungal resistance patterns, emphasizing the importance of regional diagnostic and therapeutic strategies (3, 4). The increasing frequency of

antifungal resistance, particularly to azoles such as fluconazole and voriconazole, poses a significant challenge in managing *Candida* infections. Resistance is predominantly driven by point mutations in *ERG11*, the overexpression of drug efflux pump genes, including *CDR1*, *CDR2*, and *MDR1*, as well as chromosomal amplifications that elevate resistance gene dosage (3, 5). Echinocandin resistance, once rare, is now a growing concern, particularly in *C. glabrata* and *C. auris*, with mutations in the *FKS1* and *FKS2* genes that compromise the activity of first-line agents such as anidulafungin, micafungin, and caspofungin (6, 7). Additionally, biofilm formation, a key virulence factor, provides a protected niche for fungal cells, enhancing drug tolerance and persistence, which often leads to chronic or relapsing infections (8, 9). The genomic adaptability of *Candida* further complicates treatment, with resistance arising through mechanisms such as chromosomal rearrangements, aneuploidy, loss of heterozygosity (LOH), and the formation of supernumerary chromosomes (10, 11). These changes facilitate rapid evolution under antifungal pressure, especially during prolonged or subtherapeutic exposure. Whole-genome sequencing and transcriptomic studies have revealed upregulation of stress response genes and efflux regulators, allowing *Candida* species to persist despite antifungal treatment (12, 13). Clinical isolates from recurrent infections often exhibit increased expression of heat shock proteins and resistance-related transcription factors such as *TAC1* and *UPC2*, which further exacerbate drug resistance and therapeutic failure (14, 15). Host-specific factors also influence susceptibility to *Candida* infections. Immunocompromised individuals, such as those living with human immunodeficiency virus (HIV) or undergoing chemotherapy, are at increased risk due to impaired mucosal immunity (16, 17). Pregnant women are also highly susceptible due to elevated oestrogen levels, altered vaginal pH, and immune modulation that facilitates fungal colonization and biofilm formation (18, 19). Age adds another layer of complexity, as both neonates and the elderly exhibit diminished immune responses that predispose them to candidiasis (20, 21). Moreover, racial and ethnic disparities have emerged as significant determinants of *Candida* infection outcomes. Populations including African Americans, Hispanics, and Indigenous individuals face higher infection rates and worse outcomes due to structural inequities, delayed treatment, limited healthcare access, and a higher burden of comorbidities (22-24). These disparities are especially pronounced in resource-limited regions, where diagnostic and treatment capabilities are often constrained, further enabling the spread of resistant *Candida* strains (25). In response to rising resistance, significant research has focused on developing novel antifungal therapies and alternative strategies. These include plant-derived nanoemulsions, antimicrobial peptides, probiotics, and repurposed pharmaceuticals, many of which show promise in preclinical models but require further validation before clinical integration (26-28). However, challenges such as formulation stability, strain specificity, cost, and regulatory hurdles continue to limit their widespread adoption, particularly in underserved regions. This review synthesizes current insights into the molecular, genomic, and environmental drivers of *Candida* resistance and explores both traditional and emerging therapeutic strategies. By examining the intersections of genetics, host physiology, social determinants of health, and treatment innovation, this paper aims to provide a

comprehensive understanding of the growing threat of drug-resistant *Candida* and to inform future directions in clinical and public health practice.

Global epidemiological shifts in *Candida* species

Global surveillance programs such as SENTRY and Kor-GLASS have revealed striking regional differences in the prevalence and antifungal resistance of *Candida* species. In the United States and parts of Europe, *C. glabrata* has emerged as the most common NAC species, responsible for up to 30% of bloodstream infections. It is notably resistant to fluconazole and increasingly non-susceptible to echinocandins, presenting significant therapeutic challenges (1, 7). In the Asia-Pacific region, *C. tropicalis* is the dominant NAC species, with rising fluconazole resistance and elevated minimum inhibitory concentrations (MICs) to voriconazole, which complicates the treatment of invasive infections (2). In South America and parts of Southern Europe, *C. parapsilosis* has gained attention due to the widespread presence of the *ERG11* Y132F mutation, which confers azole resistance. Its association with nosocomial transmission and clonal spread raises further concerns (29). The global rise of *C. auris* is the most alarming trend. This multidrug-resistant species is often misidentified using conventional diagnostic methods and is associated with high mortality and nosocomial outbreaks (3). In regions such as South Africa, India, and Latin America, *C. auris* isolates frequently show resistance to all four major antifungal drug classes. This highlights the urgent need for accurate identification, targeted therapy, and robust infection prevention measures (3, 4).

Resistance patterns and antifungal mechanisms in *Candida* species

Although *C. albicans* remains the most frequently isolated species, fluconazole resistance is an increasing concern, largely due to *ERG11* gene mutations that alter lanosterol 14α-demethylase and reduce azole binding affinity (5). Additional resistance mechanisms include overexpression of efflux pumps such as *CDR1*, *CDR2*, and *MDR1*, which lower intracellular drug concentrations (3). While voriconazole has shown efficacy against fluconazole-resistant *C. albicans*, cross-resistance is emerging (4). Echinocandin resistance, on the other hand, is particularly evident in *C. glabrata* and *C. auris*, where *FKS1* and *FKS2* mutations reduce susceptibility to anidulafungin, micafungin, and caspofungin (6, 7). These mutations may be accompanied by compensatory pathways that support fungal survival under echinocandin stress (11), and in *C. glabrata*, *MSH2* mutator alleles have been linked to rapid resistance acquisition (15). *C. auris* presents a global threat with over 90% of isolates resistant to fluconazole and increasing resistance to echinocandins through *FKS1* S639F mutations, particularly in intensive care settings (5, 30). It also evolves resistance rapidly via chromosomal duplications and adaptive mutations. Resistance is further enhanced by efflux pump overexpression and *ERG11* mutations such as Y132F and K143R (3). *C. glabrata* continues to show high-level fluconazole resistance and exceeds 25% in some regions, which is primarily due to *PDR1*-mediated efflux activation and hypermutability associated with *MSH2* variants (7, 15). Biofilm formation in *C. glabrata*, *C. auris*,

and *Candida haemulonii* (*C. haemulonii*) further impairs antifungal efficacy, limiting drug penetration and protecting tolerant cell populations (8). Although *C. albicans* remains generally susceptible to fluconazole, mutations in *ERG11* and increased efflux activity have led to treatment failures, especially in persistent infections (31, 32). Other NAC species have also developed significant resistance. *C. parapsilosis*, once considered fluconazole-susceptible, now shows rising resistance due to the *ERG11* Y132F mutation, with rates exceeding 30% in some settings. This mutation is also linked to clonal hospital outbreaks and co-resistance to voriconazole (29). Echinocandin resistance in *C. parapsilosis* is emerging and is associated with *FKS1* S656P mutations (1). *C. tropicalis* demonstrates increasing azole resistance in Asia and Latin America, driven by *ERG11* mutations and *UPC2*-mediated overexpression of ergosterol pathway genes (2). Molecular genotyping has confirmed clonal transmission in healthcare settings. Finally, the *C. haemulonii* species complex, including *Candida duobushaemulonii* and *Candida pseudohaemulonii,* poses a unique challenge due to intrinsic resistance to azoles and amphotericin B, as well as reduced susceptibility to echinocandins and robust biofilm formation, which complicates treatment (8). These evolving resistance patterns across *Candida* species underscore the urgency for comprehensive surveillance and antifungal stewardship.

Drug-specific factors affecting the susceptibility of *Candida* species

Antifungal agents target essential fungal processes, but resistance can arise through overlapping and drug-specific mechanisms (Figure 1). Azoles, including fluconazole and voriconazole, function by inhibiting ergosterol biosynthesis through the inhibition of lanosterol 14α-demethylase, encoded by *ERG11*. Resistance results from *ERG11* point mutations that alter the drug-binding site and reduce azole affinity (33). In addition, overexpression of efflux pumps, particularly *CDR1* and *CDR2,* lowers intracellular drug concentrations, thereby reducing azole effectiveness (34). Biofilm formation adds another dimension to azole resistance. The ECM restricts drug penetration, shielding embedded fungal cells from effective exposure and allowing them to survive treatment (35). Consequently, azoles often fail in biofilm-associated infections. Echinocandins disrupt the fungal cell wall by inhibiting β-1,3-glucan synthase. While generally effective, resistance occurs through mutations in *FKS1* and *FKS2*, which alter the catalytic domain of the enzyme, decreasing drug binding and efficacy (36). Additionally, biofilm-associated cells show reduced echinocandin susceptibility compared to their planktonic counterparts (37). Polyenes, such as amphotericin B, exert their antifungal activity by binding to ergosterol in the fungal membrane, creating pores that lead to cell death. Resistance to polyenes may arise through mutations in *ERG3*, which reduce ergosterol content and limit drug binding (38). Moreover, antioxidant enzyme production in some *Candida* strains helps neutralize reactive oxygen species generated during amphotericin B action, thereby diminishing its efficacy (34). Together, these drug-specific mechanisms demonstrate that *Candida* species can adapt their physiology and genetic expression to overcome various antifungal challenges, emphasizing the need for drug-specific susceptibility testing in clinical management.

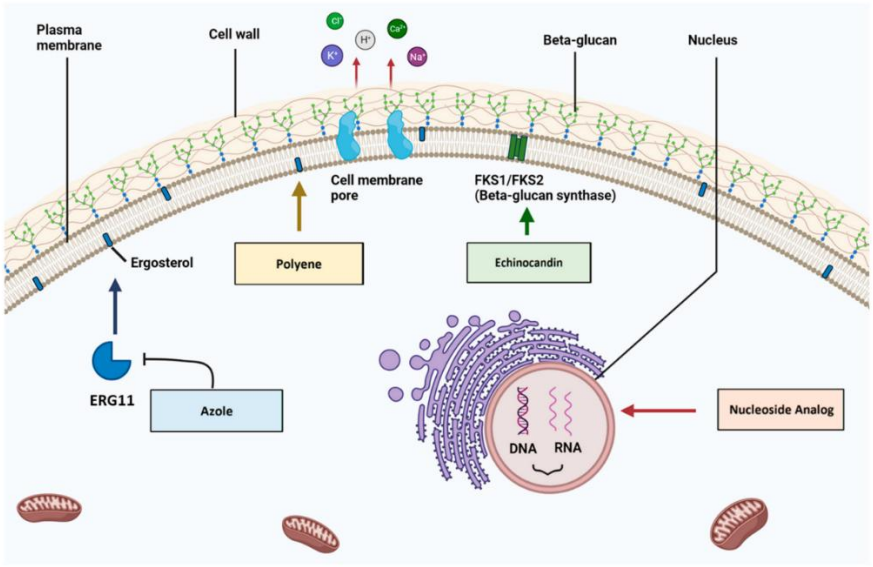

Figure 1: The four primary classes of antifungal drugs each target distinct cellular mechanisms: (I) Azoles inhibit the enzyme *ERG11*, blocking ergosterol synthesis and compromising membrane integrity. (II) Polyenes interact directly with ergosterol, forming pores in the fungal cell membrane that lead to ion leakage and cell death. (III) Echinocandins inhibit β-glucan synthase, weakening the fungal cell wall by disrupting glucan formation. (IV) Nucleoside analogues are incorporated into fungal nucleic acids, interfering with deoxyribonucleic acid (DNA) and ribonucleic acid synthesis [Adapted from *Czajka et al., 2023*] (39).

Genomic adaptability and genetic variability in *Candida* species

A defining feature of *Candida* species is their remarkable genomic plasticity, which enables rapid adaptation and results in key resistance mechanisms to antifungal drugs (Figure 2). This is particularly evident during prolonged azole exposure, where resistance arises through mechanisms such as aneuploidy, chromosomal rearrangements, and LOH, enhancing survival under stress (11). Experimental evolution studies have demonstrated that exposure to fluconazole drives the development of segmental aneuploidies, particularly involving chromosome 5, which harbours key resistance genes, including *ERG11* and *TAC1*. The duplication of these regions results in simultaneous overexpression of drug targets and resistance regulators, contributing to high-level fluconazole resistance while maintaining cellular fitness (10). In some cases, *C. albicans* develops supernumerary centric chromosomes, additional chromosomes with duplicated centromeres that confer genomic stability and resistance (10). Beyond structural variation, *Candida* species exhibit substantial genetic diversity that significantly influences antifungal susceptibility. Clinical isolates of *C. albicans* exhibit wide variability in fluconazole MICs and are often associated with *ERG11* mutations, such as Y132H and K143R. Similarly, *C. auris* frequently harbours Y132F and K143R mutations, which reduce azole binding

affinity (32, 40). Resistance also stems from *ERG11* overexpression, which is frequently driven by gain-of-function mutations in transcription factors like *UPC2*, a mechanism observed in *C. tropicalis* and *C. parapsilosis*, even in the absence of coding sequence changes (2, 29). Efflux pump overexpression further exacerbates resistance. Transcriptional activators, including *TAC1* in *C. albicans* and *PDR1* in *C. glabrata* regulate transporters such as *CDR1*, *CDR2*, and *MDR1*. Gain-of-function mutations in these regulators lead to constitutive pump activation and elevated azole MICs (15, 41). However, not all *PDR1* mutations are functionally significant, as some may be neutral or suppressive, reflecting the complexity of genetic background effects (13). Broader genomic adaptations, such as chromosomal duplications and LOH, also contribute to resistance. Amplification of chromosome 5, which includes both *ERG11* and *CDR1*, is commonly observed in fluconazole-resistant *C. albicans* (37). *C. auris* likewise demonstrates segmental duplications and large-scale rearrangements that elevate resistance to multiple antifungal classes (36, 42). LOH further supports resistance by eliminating drug-sensitive alleles. Echinocandin resistance is primarily driven by mutations in *FKS1* and *FKS2*, which encode β-1,3-D-glucan synthase. These mutations, commonly found in conserved hotspot regions, reduce the binding affinity of echinocandins. For instance, *C. glabrata* harbours F659Y and D666Y mutations in *FKS2*, while *C. auris* frequently exhibits *FKS1* S639F mutations in Clade I and III isolates and both are associated with treatment failure (7, 30, 43). Additionally, genomic instability contributes to the development of resistance. In *C. glabrata*, mutations in the DNA mismatch repair gene *MSH2* result in a hypermutator phenotype, accelerating the emergence of resistance-conferring mutations (15), although some *MSH2* variants may be neutral (44). The ABC genotyping technique is widely applied to *C. albicans*, utilizing polymerase chain reaction (PCR) amplification of the *25S ribosomal DNA* region to differentiate isolates into four genotypes: A (450 bp), B (840 bp), C (both 450 bp and 840 bp), and D (1080 bp) (45). Genotypes A, B, and C are the most frequently identified *C. albicans* genotypes with observed variations likely influenced by geographical differences (46-50). Population-level studies, including microsatellite typing and multilocus sequence typing, have revealed that different *Candida* genotypes vary not only in antifungal susceptibility but also in the expression of virulence factors (33). In *C. glabrata*, hospital outbreaks have been traced to clonal expansion of resistant genotypes (51). *C. auris* shows clade-specific susceptibility profiles; Clade I isolates are typically fluconazole-resistant, whereas Clade IV strains tend to be more susceptible (52). These findings underscore the importance of continuous molecular surveillance in informing treatment guidelines and infection control strategies.

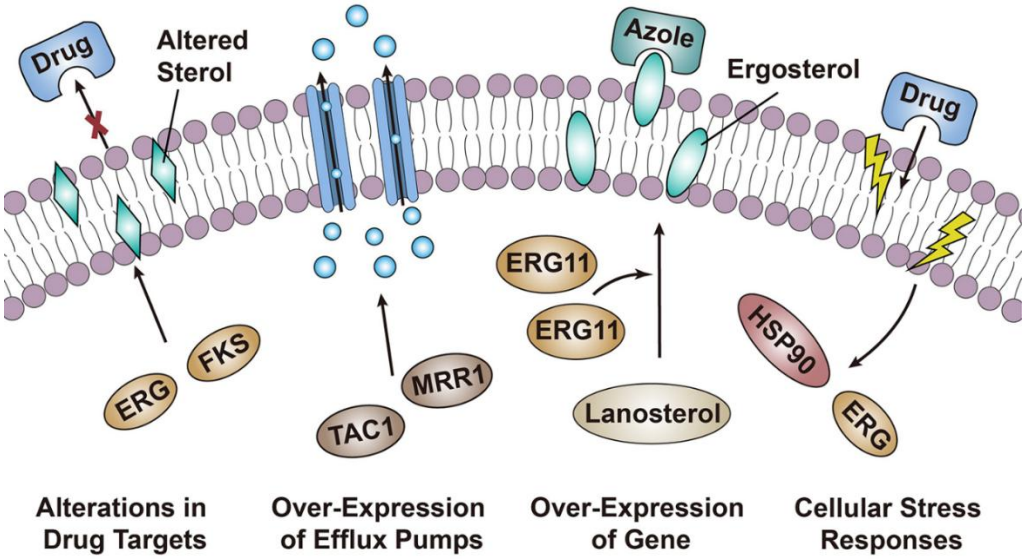

Figure 2: A schematic overview highlighting key resistance mechanisms to antifungal drugs in *C. albicans* infections. Mutations in genes such as *ERG* and *FKS* alter the corresponding drug targets, thereby reducing the efficacy of drug binding. Additionally, mutations in two critical transcription factors can lead to the upregulation of adenosine triphosphate (ATP)-binding cassette (ABC) and major facilitator transporters, resulting in the overexpression of efflux pumps that actively expel antifungal agents. Overexpression of specific genes can also increase the abundance of drug targets, such as ergosterol, thereby reducing the effectiveness of the drugs. Furthermore, antifungal-induced membrane stress can activate specific regulatory pathways and gene expression, contributing to enhanced drug tolerance [Adapted from *Gao et al., 2024*] (53).

Phenotypic mechanisms and host-related influences on antifungal resistance of *Candida* species

In addition to genetic determinants, *Candida* species exhibit phenotypic plasticity, which significantly affects their antifungal susceptibility. One of the most prominent phenotypic adaptations is biofilm formation, a key virulence trait (Figure 3). Compared to planktonic cells, biofilm-associated cells display up to 1,000-fold higher resistance to antifungals, particularly fluconazole and echinocandins (37). This is especially problematic in nosocomial infections, where biofilms form on indwelling devices, such as catheters and prosthetics (35). The biofilm extracellular matrix (ECM) (Figure 4), composed of β-glucans, mannans, proteins, and extracellular DNA, acts as both a physical and chemical barrier, sequestering drugs and limiting their penetration (9, 36). Notably, biofilm formation leads to elevated MICs across *Candida* strains regardless of their genotypes, emphasizing the importance of this phenotypic state in clinical resistance (54). While *C. auris* forms thinner biofilms than *C. albicans*, its resistance is still significant due to the presence of matrix glucans and the upregulation of efflux pumps (9). Biofilm development is also accompanied by metabolic reprogramming and transcriptional changes that enhance survival under antifungal stress. Cells within biofilms upregulate genes involved in drug efflux (*CDR1*, *CDR2*, *MDR1*), oxidative stress response, and nutrient adaptation (13, 55). Persister cells

embedded in the biofilm further complicate treatment by tolerating antifungal exposure and reseeding infection after therapy. Environmental conditions that mimic the host, such as oxidative stress, acidic pH, hypoxia, and temperature shifts, modulate antifungal responses. For example, *C. albicans* increases the expression of oxidative stress genes, such as *SOD2* and *CAP1,* in response to reactive oxygen species (13). Acidic environments, particularly those in vaginal niches, influence azole uptake and efflux activity, while nutrient limitation promotes *TAC1*-driven expression of efflux pumps. Shifts from environmental to host temperature (25°C to 37°C) affect membrane composition and susceptibility to polyenes and azoles (56). Host-related factors also shape antifungal resistance. Immunocompromised patients, such as those with HIV, undergoing chemotherapy, or organ transplantation, are especially vulnerable to persistent infections due to impaired fungal clearance (57). Certain *Candida* genotypes can alter cell wall composition to evade immune responses, further reducing drug efficacy (33). Adaptive resistance, a reversible and transient increase in MICs after sublethal drug exposure, has been observed in *C. glabrata*. It involves immediate transcriptional responses that enhance survival without permanent genetic change (58). This adds complexity to clinical treatment, especially in recurrent or biofilm-associated infections. Emerging research also highlights epigenetic and transcriptional regulatory mechanisms in antifungal tolerance. Histone modifications, such as deacetylation and methylation can influence the expression of resistance genes (59). Regulatory rewiring, such as feedback loops involving *TAC1*, *UPC2*, and *ERG11* can reinforce resistant phenotypes even in the absence of mutations. Together, these phenotypic and host-related factors create a dynamic layer of antifungal response that interacts with underlying genetic mechanisms, complicating treatment and reinforcing the need for integrated therapeutic approaches.

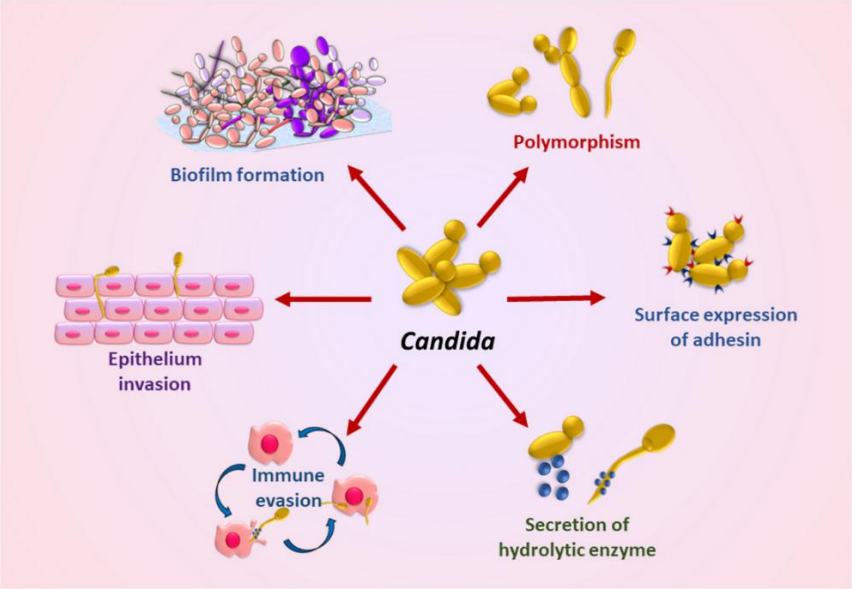

Figure 3: A visual depiction outlining the key virulence factors that contribute to *Candida*'s pathogenicity in VVC. These include its ability to switch forms (polymorphism), display adhesins on its surface, produce invasive hydrolytic enzymes, evade the immune system, and form biofilms, all of which collectively aid in its colonization and persistence [Adapted from *Chauhan et al., 2024*] (60).

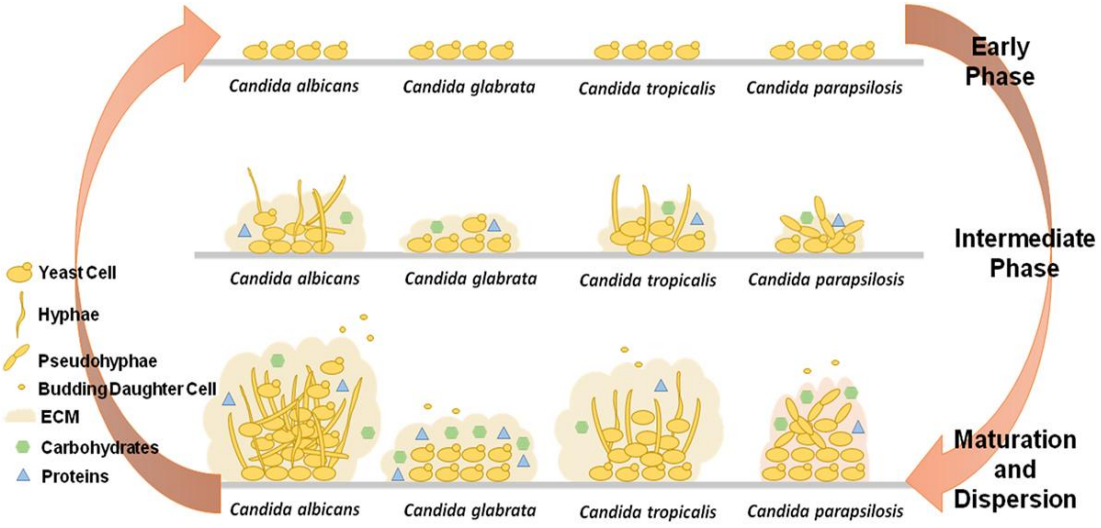

Figure 4: A comparative schematic illustrating the three distinct stages of biofilm development in *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*, emphasizing their varying abilities to produce ECM, the differences in ECM composition, and their distinct morphological transitions during biofilm formation [Adapted from *Cavalheiro et al., 2018*] (61).

Clinical, environmental, and epidemiological influences on antifungal resistance of *Candida* species

While genotypic and phenotypic characteristics are fundamental to understanding antifungal resistance in *Candida*, clinical and environmental contexts significantly influence how these pathogens respond to treatment. Factors such as drug exposure history, healthcare setting, geographic variation, and patient-specific risks collectively shape resistance patterns and treatment outcomes. Notably, the use of antifungal prophylaxis, particularly fluconazole and echinocandins, has been a major driver of resistance. Prolonged or subtherapeutic exposure selects for less susceptible genotypes, accelerating the emergence of resistance (1). In *C. glabrata*, prior azole exposure is strongly associated with *PDR1*-mediated resistance (51), while echinocandin use promotes hotspot mutations in *FKS1* and *FKS2* across both *C. glabrata* and *C. auris*. Supporting this, murine model studies have shown that gastrointestinal colonization with *C. glabrata* can rapidly acquire echinocandin resistance under therapeutic pressure, emphasizing the gut as a reservoir for resistant strains capable of causing systemic infections (15). Nosocomial candidemia, often associated with invasive interventions such as central venous catheters, mechanical ventilation, and parenteral nutrition, which creates an ideal environment for biofilm formation and resistance development (54). In intensive care units (ICUs), empirical antifungal therapy further selects for azole- or echinocandin-resistant strains, particularly among high-risk groups such as transplant recipients and neonates. In these populations, *C. parapsilosis* and *C. auris* have been frequently implicated, with evidence of clonal spread and decreased susceptibility to drugs (62). Regional antifungal use, infection control practices, and the distribution of specific clades shape geographical trends in *Candida* resistance. For example, *C. glabrata* is predominant in North America, where fluconazole resistance and rising echinocandin resistance are major concerns (1). In the Asia-Pacific region, *C. tropicalis* and *C. parapsilosis* show high azole resistance rates (2). Africa and South America have become epicentres for multidrug-resistant *C. auris* outbreaks, often involving clade-specific variants (3, 4). Interestingly, a multicentre study in Brazil identified Clade IV *C. auris* isolates that, while still azole-susceptible, exhibited traits associated with outbreak potential, highlighting the importance of continuous surveillance (52). Host factors also play a critical role in resistance dynamics. Immunosuppression, whether from chemotherapy, HIV infection, or organ transplantation diminishes fungal clearance, allowing even susceptible isolates to cause persistent infections (32). The host microbiome further modulates *Candida* colonization and the evolution of resistance. Broad-spectrum antibiotics, by disrupting bacterial competitors, facilitate fungal overgrowth and promote resistance under antifungal pressure (63). Altogether, these clinical, environmental, and epidemiological variables form an intricate framework that intersects with genetic and phenotypic traits. This synergy often leads to unpredictable outcomes, underscoring the urgent need for precision antifungal strategies that integrate fungal biology with host factors, drug pharmacodynamics, and ecological context.

HIV and *Candida*: A synergistic burden on mucosal immunity

HIV is a well-established predisposing factor for opportunistic fungal infections, particularly oropharyngeal candidiasis (OPC), oesophageal candidiasis (OEC), and VVC (64). The hallmark immunosuppression associated with HIV, especially the progressive depletion of cluster of differentiation 4-positive (CD4+) T cells, compromises mucosal immune defences and facilitates *Candida* colonization and overgrowth (17). *C. albicans* is the predominant pathogen in HIV-positive individuals and is frequently isolated from the oral cavity, gastrointestinal tract, and vaginal mucosa. Oral candidiasis often serves as an early clinical indicator of HIV progression (65, 66). The incidence of OPC and OEC increases markedly in individuals with CD4+ counts below 200 cells/µl and those with high viral loads, poor adherence to antiretroviral therapy (ART), or advanced immunosuppression (18, 67). Although ART has significantly reduced the prevalence of opportunistic fungal infections, VVC remains highly prevalent in HIV-positive women. It frequently presents as a recurrent infection and shows reduced susceptibility to first-line treatments such as fluconazole (68). Studies suggest that HIV-associated immune dysfunction alters the vaginal microbiome, reducing protective *Lactobacillus* species and promoting fungal colonization (69, 70). Furthermore, *Candida* isolates from HIV-positive patients often display enhanced biofilm formation, increased virulence, and antifungal resistance, contributing to persistent infections and therapeutic failure (71). These strains are also capable of inducing inflammatory cytokines, which can exacerbate systemic immune activation and potentially accelerate HIV disease progression (72). Co-infections and mucosal dysbiosis further complicate the clinical picture, especially in women of reproductive age, where hormonal fluctuations can impact microbial dynamics and immune responses. Given the high prevalence of antifungal resistance in HIV-associated *Candida* infections, comprehensive clinical management is essential. This includes routine surveillance, early diagnosis, and the implementation of tailored antifungal therapies. Antifungal stewardship programs and ongoing research into alternative treatments, particularly those targeting biofilm and resistance pathways, will be critical to improving outcomes in this vulnerable population (17, 64).

Pregnancy and hormonal influence on *Candida* growth

Pregnancy represents a unique physiological state characterized by profound hormonal, metabolic, and immunological changes that significantly increase susceptibility to *Candida* colonization and infection. VVC is particularly common during pregnancy, with prevalence estimates ranging from 20% to over 40%, depending on gestational age, population demographics, and geographic region (73, 74). Elevated oestrogen and progesterone levels throughout gestation play a central role in promoting *Candida* overgrowth. Oestrogen enhances glycogen deposition in the vaginal epithelium, which is metabolized into glucose and serves as a rich nutrient source for fungal proliferation (18). It also upregulates *C. albicans* adhesion, hyphal transformation, and biofilm formation. These are key steps in the establishment and persistence of infection (64, 75). Additionally, the glycogen-rich vaginal environment

during pregnancy creates ideal conditions for fungal growth and colonization. Pregnancy-induced immune modulation further contributes to this vulnerability. To maintain foetal tolerance, the maternal immune system suppresses T helper cell type 1 responses and skews toward a T helper cell type 2 dominant profile, reducing mucosal immunity and compromising antifungal defences (18, 76). While essential for foetal protection, this immunological shift inadvertently enhances susceptibility to infections, including *Candida* (77). Beyond maternal discomfort, *Candida* infections during pregnancy pose significant risks to foetal and neonatal health. VVC has been associated with preterm labour and low birth weight, likely due to fungal-induced inflammation and immune activation (19). Rare cases of intrauterine infection have been documented, resulting in neonatal invasive candidiasis (NIC) and birth complications (21). Infants exposed to untreated maternal candidiasis during vaginal delivery are at increased risk of developing early-life infections such as oropharyngeal thrush and diaper dermatitis, especially if they are premature or immunocompromised (69, 77, 78). In low-resource settings, factors such as anaemia, poor nutrition, limited healthcare access, and comorbidities like HIV further elevate the burden of VVC in pregnancy (18, 65). Given the limited number of antifungal agents deemed safe for use during pregnancy, effective management relies on early diagnosis, routine prenatal screening, and microbiome-preserving therapies to protect both maternal and neonatal health.

Age-related factors affecting the susceptibility of *Candida* species

Age is a critical determinant in susceptibility to *Candida* colonization and infection, with both ends of the age spectrum, neonates and the elderly, displaying heightened vulnerability due to immunological immaturity or decline, altered mucosal environments, and associated risk factors (20). Neonates are particularly susceptible to *Candida* infections due to their underdeveloped immune systems, thin epithelial barriers, and exposure to maternal microbiota during birth. Vertical transmission from colonized mothers during vaginal delivery can result in oral thrush, diaper dermatitis, or more severe forms of systemic candidiasis (Figure 5) (17, 79, 80). Premature and low-birth-weight infants are especially at risk, as they often require prolonged hospitalisation in neonatal intensive care units (NICUs), invasive procedures, and broad-spectrum antibiotic therapy, all of which disrupt the developing microbiome and facilitate fungal colonization. Indeed, *Candida* bloodstream infections are among the most common nosocomial fungal infections in NICUs (19, 75). In adolescents, hormonal fluctuations associated with puberty and reproductive maturation, especially in females, can predispose individuals to VVC. Contributing factors include oral contraceptive use, frequent antibiotic exposure, and early sexual activity, all of which can disrupt the vaginal microbiota and favour *Candida* overgrowth (18, 73). Elderly individuals are also at increased risk due to immunosenescence, a gradual decline in immune function marked by reduced T-cell responsiveness, impaired neutrophil activity, and weakened mucosal barrier integrity (20, 21). Comorbidities such as diabetes, cancer, and chronic pulmonary disease further compromise host defences and are associated with higher rates of OPC and biofilm-associated infections like denture stomatitis (64, 71). Medication use is another major contributor:

antibiotics, proton-pump inhibitors, and immunosuppressants frequently disrupt the microbiota, creating niches for fungal overgrowth (77, 81). Institutionalization, poor oral hygiene, and use of inhaled corticosteroids add to the burden of colonization and infection in this age group. The growing elderly population and the continued risk of NIC highlight the importance of age-specific strategies, including improved preventive care, microbiome-aware antifungal therapy, and routine screening in high-risk groups such as NICU patients and elderly individuals with chronic conditions (18).

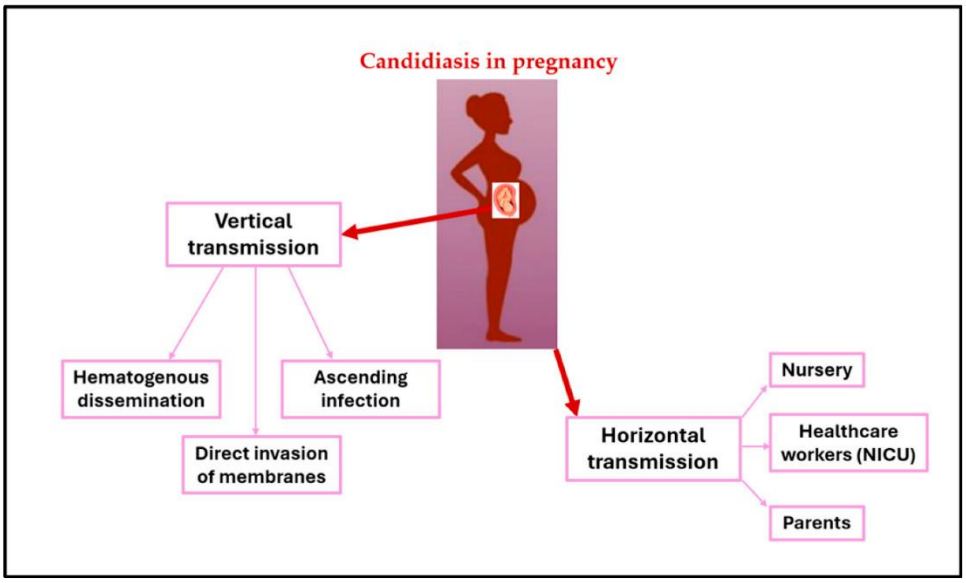

Figure 5: A visual depiction of how *Candida* spreads via vertical transmission from mother to infant during birth and through horizontal transmission between individuals or via contaminated environments [Adapted from *Messina et al., 2024*] (82).

Intersections of risk: HIV, pregnancy, and age

While HIV status, pregnancy, and age each independently heighten vulnerability to *Candida* infections, their intersection can significantly amplify risk, particularly among women of reproductive age living with HIV or aging individuals with long-term immunosuppression. These overlapping factors not only reflect biological susceptibility but also underscore deeper socioeconomic and healthcare disparities that shape infection outcomes. Pregnancy inherently alters the immune system and vaginal microbiota, creating a predisposition to *Candida* overgrowth. When coupled with HIV infection, this risk escalates dramatically, with increased incidence of VVC and OPC (65, 68). Fluconazole resistance and safety concerns during pregnancy further complicate treatment, narrowing therapeutic options in severe or recurrent cases (83). Compounding this, HIV-positive pregnant women often exhibit a vaginal microbiome deficient in *Lactobacillus*, weakening the mucosal barrier and promoting fungal colonization (67, 69). This dysbiosis is associated with adverse pregnancy outcomes such as preterm birth, low birth weight, and increased risk of NIC. In sub-Saharan Africa and other resource-limited regions, the impact is even more pronounced. Many young women experience a triple burden of high

HIV prevalence, limited maternal healthcare access, and recurrent, untreated fungal infections. The absence of routine screening and delayed access to care facilitates *Candida* persistence and vertical or sexual transmission (67). In these settings, fungal infections often go underdiagnosed and underreported, contributing to preventable complications for both mothers and infants. Although less frequently discussed, elderly individuals with HIV are usually long-term survivors on ART and represent an at-risk group. Age-related immunosenescence, comorbidities, and polypharmacy, combined with residual immune dysfunction from HIV, create conditions conducive to chronic *Candida* colonization and biofilm-related infections (20, 81). Viewing *Candida* susceptibility through an intersectional lens is essential for developing effective public health responses. Interventions must address not only isolated risk factors but also the social and demographic realities that influence access to and outcomes of health care. Tailored antifungal strategies, increased screening in high-risk populations, and integrated care for women and underserved communities are critical to controlling the spread and impact of *Candida* infections.

Emerging antifungal therapies and their limitations

The global burden of candidiasis continues to rise, driven by increasing antifungal resistance resulting from the widespread use of broad-spectrum antibiotics and the growing number of immunocompromised patients. Fluconazole, once a cornerstone of antifungal therapy, has seen a decline in efficacy, particularly in cases of recurrent vulvovaginal candidiasis (RVVC) and invasive *Candida* infections, due to the emergence of resistant strains (26, 84). Conventional antifungals, including azoles, polyenes, and echinocandins, face significant limitations. These include toxicity at higher doses, poor tissue penetration in mucosal or biofilm-associated infections, resistance mechanisms such as efflux pump activation and biofilm shielding, and low patient adherence due to prolonged treatment durations or adverse effects. These challenges underscore the urgent need for alternative therapeutic strategies that are safer, more targeted, and capable of overcoming current resistance barriers. In response, researchers are exploring bio-friendly and multifunctional therapies. Natural compounds derived from medicinal plants, functional foods, and beneficial microbes have demonstrated immunomodulatory, antimicrobial, and anti-inflammatory properties, offering promising alternatives for antifungal treatment (26, 85). Among the most notable are plant-based nanoemulsions and probiotic therapies, which have shown encouraging results in both preclinical and early clinical studies. Advances in nanotechnology have further enhanced these therapies by improving solubility, bioavailability, and targeted delivery to infection sites. Several new-generation antifungal agents are also gaining attention. Rezafungin, a novel echinocandin, has a prolonged half-life, enabling less frequent dosing while maintaining potent activity against *Candida* species (86). Oteseconazole, a next-generation azole, exhibits greater selectivity and reduced toxicity compared to fluconazole, making it a potential option for treating azole-resistant *Candida* (87). Encochleated amphotericin B is designed to minimize nephrotoxicity, one of the major drawbacks of conventional amphotericin B formulations (88).

Despite these advances, each of these novel therapies has notable shortfalls. Echinocandin resistance continues to emerge due to mutations in *FKS1*, which encodes a critical enzyme involved in fungal cell wall synthesis (28). Oteseconazole, while promising, has limited long-term safety data and a narrower spectrum of activity restricted to select *Candida* species (89). Additionally, the high cost of liposomal and encochleated amphotericin B formulations limits their accessibility in low-resource settings, where the burden of *Candida* infections is often highest (86). As resistance continues to outpace the development of new antifungals, integrated strategies combining novel agents with bio-targeted therapies, resistance monitoring, and personalized treatment regimens will be essential to curb the spread of *Candida* and improve patient outcomes globally.

Repurposing existing drugs for antifungal use against *Candida* infections

Drug repurposing has emerged as a promising and cost-effective strategy for identifying new antifungal treatments, particularly in the face of rising resistance and limited drug development pipelines. By leveraging existing pharmacological agents with known safety profiles, repurposing offers a faster route to clinical application compared to traditional drug discovery. One such agent is flucytosine, initially developed for bacterial infections. Though no longer used as monotherapy due to rapid resistance development, flucytosine has shown efficacy in combination therapies for *Candida* infections, particularly in immunocompromised patients (90). In addition, statins and certain antidepressants have been identified through high-throughput screening for their antifungal properties, demonstrating potential synergistic effects when combined with conventional antifungals (87). Despite these promising findings, repurposed drugs face several limitations. Many drugs exhibit low specificity, raising concerns about off-target effects and adverse safety profiles, particularly when used at higher doses (28). Others suffer from poor bioavailability at infection sites, especially in mucosal or biofilm-associated infections, reducing their clinical utility (90). Moreover, some repurposed agents share resistance pathways with existing antifungals, which may limit their effectiveness in treating resistant *Candida* strains (87). While drug repurposing holds clear potential, its success in antifungal therapy will depend on the development of improved delivery mechanisms, pharmacodynamic optimization, and careful resistance profiling. When strategically combined with newer antifungal agents or delivery systems, repurposed drugs could contribute meaningfully to the evolving antifungal armamentarium.

Alternative therapies against *Candida* infections: peptides, nanoparticles, and plant-based antifungals

In response to the rising threat of antifungal resistance, novel therapeutic strategies are being explored, including antimicrobial peptides (AMPs), nanotechnology-based delivery systems, and plant-derived compounds. These approaches offer promising alternatives or adjuncts to conventional antifungals, particularly in targeting biofilms and resistant *Candida* strains. AMPs such as histatins and defensins exert antifungal activity by disrupting fungal cell membranes and have demonstrated efficacy against

Candida biofilms (86). Synthetic AMPs, including modified caspofungin derivatives, are under development to improve potency and stability (87). In parallel, nanoparticles such as silver and gold have shown vigorous antifungal activity, especially against azole-resistant strains of *Candida* (88). These nanoparticles disrupt cell membranes and biofilms, offering a unique mechanism of action that complements existing therapies. Liposomal drug delivery systems enhance the bioavailability of antifungal agents and reduce systemic toxicity by targeting infection sites more precisely (28). Essential oils, including tea tree oil and thymol, have shown broad-spectrum antifungal properties and the ability to disrupt biofilm architecture (88). Additionally, certain plant-derived alkaloids exhibit synergistic effects when combined with conventional antifungals, thereby improving efficacy and potentially reducing the risk of resistance (87). However, these emerging therapies also present notable limitations. Many AMPs are rapidly degraded in the bloodstream, significantly reducing their systemic efficacy (89). Furthermore, peptide-based drugs remain costly to manufacture, which hinders their widespread use, especially in low-resource settings (86). Nanoparticle-based antifungals, while promising, require further clinical validation to assess long-term safety and potential environmental toxicity (86, 87). In the case of natural compounds, challenges include batch-to-batch variability, which complicates standardization and regulatory approval (89). Moreover, despite their *in vitro* efficacy, essential oils lack robust clinical evidence for long-term safety and effectiveness in systemic or recurrent *Candida* infections (28). Overall, while these innovative approaches hold significant promise, their successful integration into clinical practice will require continued research, standardized production, and well-designed clinical trials to address safety, efficacy, and scalability.

Plant-based nanoemulsions: a promising frontier in antifungal therapy

Plant-derived nanoemulsions have emerged as a novel and promising strategy for treating *Candida* infections, particularly in cases of drug resistance and biofilm-associated disease. These formulations incorporate bioactive phytochemicals within thermodynamically stable, nano-sized carriers, typically ranging from 20 to 200 nanometres, thereby enhancing the solubility, bioavailability, and targeted delivery of antifungal agents (91, 92). By embedding essential oils or plant-derived compounds into nanoscale emulsions, these systems exhibit multifaceted antifungal mechanisms, including the disruption of fungal cell membranes, inhibition of ergosterol synthesis (crucial for membrane integrity), and suppression of biofilm formation, which is central to *Candida* persistence and resistance (93). Their small droplet size facilitates enhanced penetration through mucosal surfaces and fungal biofilms while reducing systemic toxicity, making them especially suitable for intravaginal applications in RVVC or resistant VVC (26). Several plant nanoemulsions have demonstrated remarkable preclinical efficacy. For instance, thymol- and eugenol-based nanoemulsions effectively eradicated *C. albicans* biofilms (94), while berberine and curcumin nanoemulsions exhibited potent activity against fluconazole-resistant strains by enhancing tissue penetration and disrupting membranes (27). Cinnamon essential oil nanoemulsions not only inhibited the growth and biofilm formation of *C. albicans* but also reduced

inflammation in murine vaginal candidiasis models (95). More advanced systems, such as silver-secnidazole hybrid nanoemulsions combined with probiotics, demonstrated synergistic effects by destroying fungal membranes and enhancing *Lactobacillus* colonization, supporting both pathogen clearance and microbiome restoration (26). Porphyra oligosaccharide-based nanoemulsions further stimulated mucosal immunity and restored healthy vaginal flora, offering dual antifungal and immunomodulatory effects (85). Compared to conventional azoles and polyenes, plant-based nanoemulsions provide several advantages, including lower irritation and toxicity, enhanced biofilm penetration, a reduced likelihood of resistance development, and potential for use in combination therapies. These properties make them highly attractive candidates for localized drug delivery, particularly for patients experiencing chronic or recurrent infections. However, despite their promise, several limitations remain. Most efficacy data are derived from *in vitro* studies or animal models, with limited clinical trials validating long-term safety and effectiveness in humans (92). Stability can also be a concern; nanoemulsions require specific stabilizers to prevent degradation over time (96). Furthermore, variability in plant sources and extraction techniques can affect consistency and standardization (97). These challenges must be addressed before nanoemulsions can be widely adopted in clinical practice. Nonetheless, plant nanoemulsions represent an exciting direction for antifungal innovation, bridging traditional herbal therapeutics with cutting-edge nanomedicine to combat the growing threat of *Candida* resistance.

Probiotics (*Lactobacillus* species) as an antifungal approach

Probiotics, particularly strains of *Lactobacillus*, are emerging as valuable adjuncts or alternatives to conventional antifungal therapies, offering a bio-friendly approach to managing *Candida* infections. As natural residents of the healthy vaginal microbiota, *Lactobacillus* species play a pivotal role in maintaining microbial balance and preventing pathogenic overgrowth (Figure 6) (85, 98). The antifungal activity of probiotics is multifactorial, involving competition for adhesion sites on epithelial cells, which reduces *Candida* colonization. The production of antimicrobial compounds such as lactic acid, hydrogen peroxide, and bacteriocins, which inhibit fungal growth (99, 100), and modulation of host immunity, promoting mucosal defences and antifungal immune responses (101). Strains such as *Lactobacillus rhamnosus* (*L. rhamnosus*), *Lactobacillus reuteri* (*L. reuteri*), *Lactobacillus crispatus*, and *Lactobacillus acidophilus* have demonstrated anti-*Candida* effects both *in vitro* and *in vivo*, including inhibition of hyphal formation, suppression of biofilm development, and disruption of established fungal communities (101). Clinical studies further support the therapeutic value of probiotics. For example, a survey of VVC showed that the combination of *L. rhamnosus* and fluconazole significantly reduced recurrence rates compared to fluconazole alone (102). Probiotic supplementation, whether oral or intravaginal, has been shown to enhance the efficacy of azoles and plant-based antifungals, support the re-establishment of healthy vaginal flora following antifungal treatment, and reduce recurrence of VVC while improving overall treatment outcomes. Emerging delivery systems,

such as probiotic-loaded nanoformulations and dual-action therapies combining probiotics with antifungal agents, are being developed to increase mucosal colonization and prolong therapeutic effects (26). These innovative formulations aim to prevent relapse and overcome challenges associated with standard antifungal treatments. Probiotic therapies are particularly relevant for immunocompromised populations, including individuals with HIV, who often experience vaginal microbiome dysbiosis that predisposes them to *Candida* colonization. Reintroducing protective *Lactobacillus* strains has shown promise in restoring microbial equilibrium and reducing opportunistic fungal infections (99). However, several limitations remain. Not all *Lactobacillus* strains possess antifungal properties, meaning strain selection is critical (100). Many probiotics fail to establish long-term residency in the host, requiring repeated administration (101). Additionally, while rare, some probiotic strains may act as opportunistic pathogens in immunocompromised individuals, highlighting the importance of rigorous safety screening (100). Despite these challenges, probiotics represent a promising and adaptable tool in the fight against *Candida*, particularly when integrated into multifaceted treatment strategies tailored to individual patient profiles.

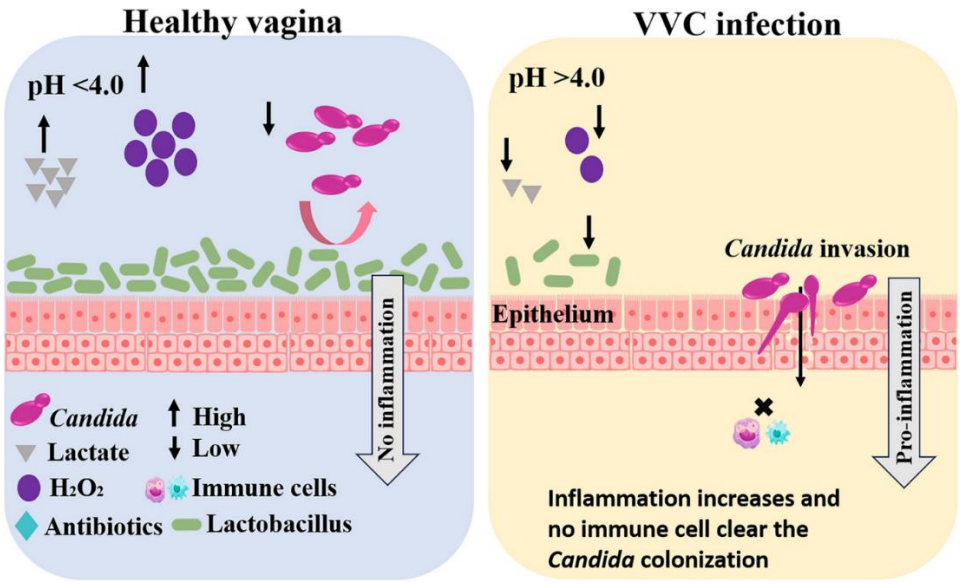

Figure 6: An illustration showing how naturally occurring *Lactobacillus* species in the vaginal microbiota help regulate *Candida* colonization by maintaining an acidic environment with low pH and elevated lactate levels [Adapted from *Chauhan et al., 2024*] (60).

Shortfalls and limitations of alternative therapies

Developing new antifungal drugs is both costly and time-consuming, with limited financial incentives for pharmaceutical companies to invest in antifungal research and development (90). Regulatory approval processes add further delays, slowing access to innovative therapies even as the burden of fungal resistance continues to grow (87). *Candida* species pose a significant challenge due to their diverse and overlapping resistance mechanisms, including the activation of efflux pumps, robust biofilm

formation, and rapid genetic mutation (28). Alarmingly, resistance is already emerging against several next-generation antifungals, underscoring the urgent need for combination therapies and alternative treatment strategies (88). Although plant-based nanoemulsions and probiotic therapies offer promising antifungal potential, their integration into mainstream treatment remains hindered by several scientific, clinical, and logistical barriers. For nanoemulsions, issues related to stability and shelf life remain significant, as many plant-derived bioactives are sensitive to environmental factors such as light, pH, and temperature, which can potentially compromise their integrity over time (91). Additionally, standardization is challenging due to variability in essential oil composition, which is influenced by species, extraction methods, and geographic origin (103). Most studies on antifungal nanoemulsions remain in the preclinical stage, and their efficacy and safety in large-scale human trials have yet to be validated. Furthermore, their application is limited mainly to topical or mucosal surfaces, reducing their utility in treating invasive candidiasis or systemic infections (104). Probiotic therapies face similarly complex limitations. Their antifungal efficacy is highly strain-dependent, and not all *Lactobacillus* species demonstrate consistent anti-*Candida* activity across populations or infection types (85, 99). Colonization tends to be transient, requiring ongoing supplementation, and many probiotic strains struggle to survive the manufacturing process, long-term storage, or passage through the gastrointestinal tract. Additionally, regulatory frameworks in many countries classify probiotics as dietary supplements rather than therapeutic agents, which limits clinical oversight and makes claims of efficacy difficult to substantiate. The broader adoption of these alternative therapies is also hindered by high production costs, regulatory barriers, patient compliance challenges, particularly with combination or prolonged regimens, and limited access in low-resource settings. To advance these therapies toward clinical implementation, coordinated efforts are needed to support well-designed clinical trials, standardize formulations, and integrate probiotic and nanoemulsion-based interventions into comprehensive, patient-centred care models.

Epidemiology of *Candida* infections across racial and ethnic groups

Epidemiological data reveal a concerning pattern: certain racial and ethnic groups consistently experience a disproportionately higher incidence, severity, and mortality from both superficial and systemic *Candida* infections. These disparities are most pronounced among African American, Hispanic or Latino, Indigenous, and Southeast Asian populations. In the United States, African American patients are particularly affected by candidemia. A large population-based study by *Cleveland et al. (2015)* found that Black individuals had nearly twice the candidemia incidence rate than white patients (105). These findings were echoed in more recent studies, including one conducted in California, which reported persistently higher rates of invasive *Candida* infections in Black patients, even after adjusting for comorbidities and ICU exposure (24). Contributing factors include higher prevalence of diabetes and HIV as well as increased exposure to central venous catheters in ICU settings. Delayed initiation of antifungal therapy is often a reflection of structural inequities in healthcare access (23, 25, 106). Racial

bias in healthcare systems has also been implicated in delayed diagnosis and under-treatment among minority groups (25, 107). Hispanic and Latino individuals similarly exhibit elevated vulnerability to both mucosal and systemic *Candida* infections. VVC is especially prevalent among pregnant Latina women, driven by hormonal shifts, antibiotic exposure, and limited access to prenatal care and gynaecological education (108). In a multicentre candidemia study, Hispanic patients experienced longer hospital stays, higher readmission rates, and more treatment delays, especially among those without adequate health insurance (109). Indigenous populations across both high- and low-income countries are also disproportionately affected by *Candida* infections. In rural and reservation-based settings, underdiagnosis and undertreatment are common due to infrastructure deficits and lack of access to specialized care. Among Australian Aboriginal and Canadian First Nations communities, OPC and systemic fungal infections occur at rates well above national averages, particularly among immunocompromised individuals (110-112). Similar trends are observed in Southeast Asia and South Asia, where studies from India, Pakistan, and urban slums across the region report high prevalence of both *C. albicans* and NAC species in women of reproductive age. Limited access to antifungal medications, widespread antibiotic overuse, and empiric treatment practices contribute to the recurrence and rising resistance of infections (113, 114). Migrant populations, including recent immigrants and refugees, are particularly vulnerable due to poor housing, lack of insurance, language barriers, and delayed healthcare engagement. One study found significantly higher rates of oral and gastrointestinal *Candida* colonization among recent immigrants, with misdiagnosis and lack of treatment common due to systemic access issues (106). Additionally, Native American communities face unique risks, including a higher incidence of severe complications like fungal endophthalmitis and limited availability of specialized fungal care services (112). Occupational exposure to environmental fungi, especially in agricultural and food-processing industries, may also elevate infection risk in Hispanic and Indigenous populations (23). Collectively, these findings highlight the urgent need for culturally competent, accessible, and equitable antifungal care tailored to underserved and disproportionately affected populations.

Risk factors behind racial disparities in *Candida* susceptibility

The elevated burden of *Candida* infections in specific racial and ethnic groups cannot be fully explained by biological susceptibility alone. These disparities are multifactorial and rooted in a complex interplay between socioeconomic inequities, healthcare access limitations, prevalent comorbidities, environmental exposures, and behavioural practices. Access to timely and high-quality healthcare remains one of the most significant determinants of *Candida* outcomes. Minority populations, including Black, Hispanic or Latino, Indigenous, and migrant groups, are more likely to experience limited access to primary care, underinsurance or lack of health insurance, delayed initiation of antifungal therapy, and reduced access to diagnostic tools such as fungal cultures or PCR testing (24, 106). These structural barriers lead to delayed treatment, increased disease severity, and higher mortality rates, particularly in

cases of candidemia (23). Rural and underserved urban communities, which are frequently home to Black, Indigenous, or Hispanic populations, also face longer hospital response times and shortages of infectious disease specialists, compounding delays in diagnosis and care (25, 115). Comorbid conditions that increase susceptibility to *Candida* are disproportionately prevalent in minority groups. Type 2 diabetes, for example, is more common among African American and Hispanic individuals and is associated with increased rates of mucosal *Candida* colonization and infection (116). HIV remains more prevalent in Black and Hispanic communities in the United States, placing these groups at heightened risk for OPC and OEC (117). Pregnant women of colour, particularly Latina and African women, report higher rates of VVC, often due to hormonal changes, antibiotic exposure, and limited access to prenatal education and care (118). Immunosuppressive therapies such as corticosteroids, usually used in transplant or chronic disease management, are also more frequently administered to minority patients with complex health profiles, further increasing *Candida* vulnerability (24, 25). Environmental exposures compound these risks. Poor housing conditions, overcrowding, inadequate sanitation infrastructure, and Mold exposure are more prevalent in communities of colour and among migrant or refugee populations, creating favourable conditions for *Candida* overgrowth and persistence (23, 106). In long-term care settings, elderly patients of colour face unique risks for oral candidiasis, often linked to infrequent denture cleaning, xerostomia, and limited access to dental care (111). Occupational exposure also plays a role in Black and Hispanic individuals who are overrepresented in agricultural, food-processing, and urban industrial jobs where fungal contamination is common (23). Additionally, increased airborne fungal exposure in densely populated urban areas has been associated with elevated *Candida* colonization rates (108). Behavioural and cultural factors, including mistrust of medical institutions, reliance on traditional remedies, or language barriers, may also delay healthcare-seeking behaviour or contribute to incomplete or inappropriate treatment, potentially worsening resistance patterns and outcomes (113). While emerging evidence suggests that host immunity and microbiota composition may vary across ethnic groups, genetic susceptibility data remain limited and inconclusive. The majority of disparities appear to stem from modifiable, non-genetic factors, offering a powerful opportunity for targeted public health interventions aimed at reducing *Candida* burden in historically underserved populations (13).

Integrative strategies to combat drug-resistant *Candida* species: clinical, public health, and equity-focused approaches

The global rise in fluconazole-resistant *C. albicans* presents a critical public health challenge, especially in resource-limited settings where azoles remain the primary antifungal agents (32). Resistance mechanisms complicate the treatment of recurrent infections such as VVC, candidemia, and device-associated biofilm infections. Biofilm-forming genotypes, particularly A and C, often require alternative therapies, such as echinocandins or amphotericin B, which are cost-prohibitive in many regions (119). To address these challenges, clinical strategies must prioritize routine antifungal susceptibility testing

and genotypic profiling of clinical isolates for early detection of resistance (120). Adjunctive therapies targeting biofilm dispersal, efflux pump inhibition, and fungal stress responses are being explored, while probiotic-based treatments offer potential for restoring microbial balance and enhancing host immunity (71). Resistance arises from the convergence of genetic mutations (e.g., *ERG11*, *PDR1*, *FKS1*), phenotypic adaptations such as biofilm formation, and external pressures, including antifungal exposure and regional clade prevalence (30, 42). Precision diagnostics such as molecular assays and genome sequencing are indispensable for targeted therapy and outbreak control. For instance, identifying *FKS* mutations in *C. glabrata* or *C. auris* can justify the early use of amphotericin B or next-generation echinocandins, such as rezafungin (1). Management of biofilm-related infections may also require catheter removal or antifungal lock therapy. Proactive screening of transplant recipients, neonates, and immunocompromised patients can help prevent the spread of resistant clades, such as *C. auris* Clade I and IV (3, 52). Novel antifungals, such as manogepix and ibrexafungerp are promising against multidrug-resistant strains, while combination therapy using echinocandins and azoles or flucytosine has demonstrated synergistic effects (121). Anti-virulence strategies targeting biofilm matrix or host modulation are also emerging (9). Cutting-edge tools like artificial intelligence, systems biology, and machine learning are being applied to predict resistance dynamics and guide stewardship (13). HIV status, pregnancy, and age significantly influence *Candida* colonization and infection risk, requiring personalized prevention and screening particularly among HIV-positive individuals, pregnant women, and the elderly (18, 73). In high-burden populations like HIV-positive young women in sub-Saharan Africa, integrating fungal care into reproductive health initiatives and promoting hygiene and ART adherence are critical (18, 67). Rising resistance demands enhanced surveillance, stewardship, and antifungal testing in clinical laboratories (70). For HIV-positive pregnant women, a dual-care model involving obstetricians and infectious disease specialists ensures safe antifungal use (122). Probiotics are also gaining traction as adjuncts for recurrent VVC, particularly in pregnant and non-pregnant women (71). To translate alternative therapies, such as nanoemulsions and probiotics, into clinical use, robust trials, good manufacturing practices, and adherence to regulatory standards are essential (85, 91). Optimizing probiotic strains, dosing, and delivery methods will enhance treatment outcomes. Combination strategies, such as chitosan-coated nanoparticles, dual-loaded systems, or the synergistic pairing of antifungals with probiotics, show potential for reducing recurrence and resistance (26). In low-resource settings, affordability and cultural relevance are critical. Empowering communities through education, local production, and the integration of traditional knowledge can enhance access and impact. Reducing racial and ethnic disparities in *Candida* care requires community-based programs, multilingual health services, subsidized medications, and cultural competency training for clinicians (23-25). Inclusive care improves diagnosis and outcomes, particularly for patients with diabetes, HIV, and pregnancy-related immunosuppression (107, 110). Mobile clinics and PCR diagnostics offer early detection in underserved communities (112), while workplace safety regulations and protective strategies can reduce occupational exposure (23). Educational outreach tailored to Indigenous and rural

populations improves awareness and lowers preventable infections (111, 118). Preventative strategies, including topical prophylaxis, antifungal rinses, and probiotic supplementation, show efficacy in reducing VVC recurrence (106, 114). Nutritional support may also enhance immune function. Long-term success hinges on investments in diagnostics, antifungal stewardship, standardized reporting, and healthcare infrastructure in minority-serving hospitals (1). Probiotics, such as *L. reuteri* and *L. rhamnosus*, show promise in restoring vaginal balance, although clinical outcomes remain inconsistent (102). Ibrexafungerp, a novel triterpenoid antifungal, has demonstrated efficacy against RVVC and is under further evaluation (25). Although maintenance therapies like fluconazole or boric acid help reduce recurrence, resistance, and toxicity risks necessitate more sustainable options (123). A multifaceted approach that blends novel therapeutics, community engagement, and systemic reform is essential to overcoming the complex threat of antifungal resistance.

Conclusion

Antifungal resistance among *Candida* species is a rapidly evolving challenge that demands urgent, integrated action across molecular science, clinical medicine, and public health. The emergence of resistant NAC species, particularly *C. auris*, *C. glabrata*, and *C. parapsilosis*, highlights the complexity of resistance mechanisms, ranging from genetic mutations and efflux pump overexpression to biofilm formation and chromosomal adaptation. These traits allow *Candida* to thrive despite antifungal pressure, making conventional therapies less effective and increasing the risk of persistent or recurrent infection. This review has highlighted how resistance is not only driven by molecular and environmental factors but is also deeply shaped by host-specific vulnerabilities. Conditions such as HIV infection, pregnancy, and immunosenescence in the elderly significantly increase *Candida* colonization and disease burden. Additionally, social determinants of health, including race, ethnicity, access to care, and geographic location, contribute to wide disparities in diagnosis, treatment, and outcomes. Populations historically marginalized in healthcare systems continue to experience disproportionate rates of infection, under-treatment, and mortality. In the face of these challenges, emerging antifungal strategies, including plant nanoemulsions, probiotics, peptides, and repurposed drugs, offer significant promise. However, these must overcome clinical and regulatory barriers to achieve widespread adoption. The integration of novel therapies with diagnostic innovations, resistance profiling, and antifungal stewardship programs is essential for improving outcomes. Furthermore, inclusive and culturally competent care models are critical to addressing the intersection of biological susceptibility and structural inequities. Ultimately, controlling the spread and impact of drug-resistant *Candida* will require sustained investment in research, diagnostics, equity-focused public health strategies, and collaborative clinical care. Only through a multifaceted and holistic approach can we begin to reverse the global trajectory of antifungal resistance and protect vulnerable populations worldwide.

Author Contributions

Caitlin Ramnarain: conceptualization, visualization, writing - original draft. **Nathlee Abbai:** visualization, supervision, writing - review and editing. **Refilwe Phemelo Molatlhegi:** conceptualization, supervision, writing - review and editing.

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Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

References

1. Pfaller MA, Diekema DJ, Turnidge JD, Castanheira M, Jones RN. Twenty Years of the SENTRY Antifungal Surveillance Program: Results for Candida Species From 1997–2016. Open Forum Infectious Diseases. 2019;6(Supplement_1):S79-S94.

2. Arastehfar A, Daneshnia F, Hafez A, Khodavaisy S, Najafzadeh M-J, Charsizadeh A, et al. Antifungal susceptibility, genotyping, resistance mechanism, and clinical profile of Candida tropicalis blood isolates. Medical Mycology. 2020;58(6):766-73.

3. Jacobs SE, Jacobs JL, Dennis EK, Taimur S, Rana M, Patel D, et al. Candida auris Pan-Drug-Resistant to Four Classes of Antifungal Agents. Antimicrob Agents Chemother. 2022;66(7):e0005322.

4. Maphanga TG, Naicker SD, Kwenda S, Muñoz JF, Schalkwyk Ev, Wadula J, et al. In Vitro Antifungal Resistance of Candida auris Isolates from Bloodstream Infections, South Africa. Antimicrobial Agents and Chemotherapy. 2021;65(9):10.1128/aac.00517-21.

5. Rybak JM, Muñoz JF, Barker KS, Parker JE, Esquivel BD, Berkow EL, et al. Mutations in TAC1B: a Novel Genetic Determinant of Clinical Fluconazole Resistance in Candida auris. mBio. 2020;11(3):10.1128/mbio.00365-20.

6. Aldardeer NF, Albar H, Al-Attas M, Eldali A, Qutub M, Hassanien A, et al. Antifungal resistance in patients with Candidaemia: a retrospective cohort study. BMC infectious diseases. 2020;20:1-7.

7. Khalifa HO, Arai T, Majima H, Watanabe A, Kamei K. Genetic Basis of Azole and Echinocandin Resistance in Clinical Candida glabrata in Japan. Antimicrob Agents Chemother. 2020;64(9).

8. Kotey FC, Dayie NT, Tetteh-Uarcoo PB, Donkor ES. Candida bloodstream infections: changes in epidemiology and increase in drug resistance. Infectious Diseases: Research and Treatment. 2021;14:11786337211026927.

9. Dominguez EG, Zarnowski R, Choy HL, Zhao M, Sanchez H, Nett JE, et al. Conserved Role for Biofilm Matrix Polysaccharides in Candida auris Drug Resistance. mSphere. 2019;4(1):10.1128/mspheredirect.00680-18.

10. Narayanan A, Kumar P, Chauhan A, Kumar M, Yadav K, Banerjee A, et al. Directed evolution detects supernumerary centric chromosomes conferring resistance to azoles in Candida auris. MBio. 2022;13(6):e03052-22.

11. Ksiezopolska E, Schikora-Tamarit MÀ, Beyer R, Nunez-Rodriguez JC, Schüller C, Gabaldón T. Narrow mutational signatures drive acquisition of multidrug resistance in the fungal pathogen Candida glabrata. Current Biology. 2021;31(23):5314-26. e10.

12. Kim SH, Iyer KR, Pardeshi L, Muñoz JF, Robbins N, Cuomo CA, et al. Genetic Analysis of Candida auris Implicates Hsp90 in Morphogenesis and Azole Tolerance and Cdr1 in Azole Resistance. mBio. 2019;10(1):10.1128/mbio.02529-18.

13. Xu Y, Lu H, Zhu S, Li W-Q, Jiang Y-y, Berman J, et al. Multifactorial mechanisms of tolerance to ketoconazole in Candida albicans. Microbiology Spectrum. 2021;9(1):10.1128/spectrum. 00321-21.

14. Pais P, Califórnia R, Galocha M, Viana R, Ola M, Cavalheiro M, et al. Candida glabrata transcription factor Rpn4 mediates fluconazole resistance through regulation of ergosterol biosynthesis and plasma membrane permeability. Antimicrobial Agents and Chemotherapy. 2020;64(9):10.1128/aac. 00554-20.

15. Healey KR, Zhao Y, Perez WB, Lockhart SR, Sobel JD, Farmakiotis D, et al. Prevalent mutator genotype identified in fungal pathogen Candida glabrata promotes multi-drug resistance. Nature communications. 2016;7(1):11128.

16. Pankuch GA, Appelbaum PC. Postantibiotic Effect of Ceftobiprole against 12 Gram-Positive Organisms. Antimicrobial Agents and Chemotherapy. 2006;50(11):3956-8.

17. McKloud E, Delaney C, Sherry L, Kean R, Williams S, Metcalfe R, et al. Recurrent Vulvovaginal Candidiasis: a Dynamic Interkingdom Biofilm Disease of Candida and Lactobacillus. mSystems. 2021;6(4):e0062221.
18. Payne VK, Florence Cecile TT, Cedric Y, Christelle Nadia NA, Jose O. Risk Factors Associated with Prevalence of Candida albicans, Gardnerella vaginalis, and Trichomonas vaginalis among Women at the District Hospital of Dschang, West Region, Cameroon. Int J Microbiol. 2020;2020:8841709.
19. Nisha D, Fahmitha FHF, Kaviya G, Padmavathi V, Vijay K. Global Clinical Case Studies in Candida species: A Review. Asian Journal of Research in Biosciences. 2024;6(2):143-56.
20. Brown GD, Denning DW, Levitz SM. Tackling Human Fungal Infections. Science. 2012;336(6082):647-.
21. Giacobbe DR, Laura M, Chiara S, Malgorzata M, Philipp K, A. CO, et al. Recent advances and future perspectives in the pharmacological treatment of Candida auris infections. Expert Review of Clinical Pharmacology. 2021;14(10):1205-20.
22. Toda M WS, Berkow EL, et al. Population-Based Active Surveillance for Culture-Confirmed Candidemia — Four Sites, United States, 2012–2016. MMWR Surveill Summ. 2019;68(No. SS-8):1–15.
23. Seidelman J, Fleece M, Bloom A, Lydon E, Yang W, Arnold C, et al. Endogenous Candida endophthalmitis: Who is really at risk? Journal of Infection. 2021;82(2):276-81.
24. Grant VC, Zhou AY, Tan KK, Abdul-Mutakabbir JC. Racial disparities among candidemic patients at a Southern California teaching hospital. Infection Control & Hospital Epidemiology. 2023;44(11):1866-9.
25. Nsenga L, Bongomin F. Recurrent Candida Vulvovaginitis. Venereology. 2022;1(1):114-23.
26. Mosallam FM, Helmy EA, El-Bastawisy HS, El-Batal AI. Silver secnidazole nano-hybrid emulsion-based probiotics as a novel antifungal formula against multidrug-resistant vaginal pathogens. Biotechnology and Applied Biochemistry. 2024;72(2):295-310.
27. Donadu MG, D. U, M. M, M. U, V. M, P. M, et al. Antifungal activity of oils macerates of North Sardinia plants against Candida species isolated from clinical patients with candidiasis. Natural Product Research. 2020;34(22):3280-4.
28. Stewart AG, and Paterson DL. How urgent is the need for new antifungals? Expert Opinion on Pharmacotherapy. 2021;22(14):1857-70.
29. Papp C, Bohner F, Kocsis K, Varga M, Szekeres A, Bodai L, et al. Triazole Evolution of Candida parapsilosis Results in Cross-Resistance to Other Antifungal Drugs, Influences Stress Responses, and Alters Virulence in an Antifungal Drug-Dependent Manner. mSphere. 2020;5(5):10.1128/msphere.00821-20.
30. Kordalewska M, Lee A, Park S, Berrio I, Chowdhary A, Zhao Y, et al. Understanding echinocandin resistance in the emerging pathogen Candida auris. Antimicrobial agents and chemotherapy. 2018;62(6):10.1128/aac. 00238-18.

31. Xiao M, Chen SC, Kong F, Xu XL, Yan L, Kong HS, et al. Distribution and Antifungal Susceptibility of Candida Species Causing Candidemia in China: An Update From the CHIF-NET Study. J Infect Dis. 2020;221(Suppl 2):S139-s47.
32. Sanguinetti M, Posteraro B, Lass-Flörl C. Antifungal drug resistance among Candida species: mechanisms and clinical impact. Mycoses. 2015;58(S2):2-13.
33. Wang M, Cao Y, Xia M, Al-Hatmi AM, Ou W, Wang Y, et al. Virulence and antifungal susceptibility of microsatellite genotypes of Candida albicans from superficial and deep locations. Yeast. 2019;36(5):363-73.
34. Danielly BdSS, Alexéia BG, Kelly MPdO. Genetic determinants of antifungal resistance in Candida species. African Journal of Biotechnology. 2016;15(40):2259-64.
35. Liu X, Fan S, Bai F, Li J, Liao Q. Antifungal susceptibility and genotypes of Candida albicans strains from patients with vulvovaginal candidiasis. Mycoses. 2009;52(1):24-8.
36. Kwon YJ, Shin JH, Byun SA, Choi MJ, Won EJ, Lee D, et al. Candida auris Clinical Isolates from South Korea: Identification, Antifungal Susceptibility, and Genotyping. Journal of Clinical Microbiology. 2019;57(4):10.1128/jcm.01624-18.
37. McCullough MJ, Clemons KV, Stevens DA. Molecular and phenotypic characterization of genotypic Candida albicans subgroups and comparison with Candida dubliniensis and Candida stellatoidea. Journal of clinical microbiology. 1999;37(2):417-21.
38. Carolus H, Pierson S, Lagrou K, Van Dijck P. Amphotericin B and Other Polyenes—Discovery, Clinical Use, Mode of Action and Drug Resistance. Journal of Fungi. 2020;6(4):321.
39. Czajka KM, Venkataraman K, Brabant-Kirwan D, Santi SA, Verschoor C, Appanna VD, et al. Molecular Mechanisms Associated with Antifungal Resistance in Pathogenic Candida Species. Cells. 2023;12(22):2655.
40. Li J, Coste AT, Liechti M, Bachmann D, Sanglard D, Lamoth F. Novel ERG11 and TAC1b Mutations Associated with Azole Resistance in Candida auris. Antimicrobial Agents and Chemotherapy. 2021;65(5):10.1128/aac.02663-20.
41. Schneider S, Morschhäuser J. Induction of Candida albicans drug resistance genes by hybrid zinc cluster transcription factors. Antimicrobial Agents and Chemotherapy. 2015;59(1):558-69.
42. Carolus H, Pierson S, Muñoz JF, Subotić A, Cruz RB, Cuomo CA, et al. Genome-wide analysis of experimentally evolved Candida auris reveals multiple novel mechanisms of multidrug resistance. MBio. 2021;12(2):10.1128/mbio. 03333-20.
43. Sharma D, Paul RA, Rudramurthy SM, Kashyap N, Bhattacharya S, Soman R, et al. Impact of FKS1 genotype on echinocandin in vitro susceptibility in Candida auris and in vivo response in a murine model of infection. Antimicrobial agents and chemotherapy. 2022;66(1):e01652-21.
44. Bordallo-Cardona MÁ, Agnelli C, Gómez-Nuñez A, Sánchez-Carrillo C, Bouza E, Muñoz P, et al. MSH2 gene point mutations are not antifungal resistance markers in Candida glabrata. Antimicrobial Agents and Chemotherapy. 2019;63(1):10.1128/aac. 01876-18.

45. Fornari G, Vicente VA, Gomes RR, Muro MD, Pinheiro RL, Ferrari C, et al. Susceptibility and molecular characterization of Candida species from patients with vulvovaginitis. brazilian journal of microbiology. 2016;47:373-80.
46. Shekhany KAM. Isolation and genotyping of Candida albicans involved in vaginal candidiasis among pregnant women in Sulaymaniyah and Erbil cities. Zanco Journal of Medical Sciences (Zanco J Med Sci). 2021;25(1):493-502.
47. Noori AM, Bander K, Hamada T. Genotype Comparisons of Candida albicans From Patients With Vulvovaginal Candidiasis. Egyptian Academic Journal of Biological Sciences, G Microbiology. 2016;8(1):1-5.
48. Araújo Paulo de Medeiros M, Vieira de Melo AP, Gonçalves SS, Milan EP, Chaves GM. Genetic relatedness among vaginal and anal isolates of Candida albicans from women with vulvovaginal candidiasis in north-east Brazil. Journal of medical microbiology. 2014;63(11):1436-45.
49. Ali-Shtayeh M, Jamous R, Alothman N, Baker MA, Zaitoun SA, Mallah O, et al. Genotyping and antifungal susceptibility of Candida albicans strains from patients with vulvovaginal and cutaneous candidiasis in Palestine. 2015.
50. Gharaghani M, Shabanzadeh M, Jafarian H, Zarei Mahmoudabadi A. ABC typing and extracellular enzyme production of Candida albicans isolated from Candida vulvovaginitis. Journal of Clinical Laboratory Analysis. 2022;36(1):e24117.
51. Goemaere B, Lagrou K, Spriet I, Hendrickx M, Becker P. Clonal spread of Candida glabrata bloodstream isolates and fluconazole resistance affected by prolonged exposure: a 12-year single-center study in Belgium. Antimicrobial Agents and Chemotherapy. 2018;62(8):10.1128/aac. 00591-18.
52. Spruijtenburg B, Nobrega de Almeida Júnior J, Ribeiro FdC, Kemmerich KK, Baeta K, Meijer EF, et al. Multicenter Candida auris outbreak caused by azole-susceptible clade IV in Pernambuco, Brazil. Mycoses. 2024;67(6):e13752.
53. Gao Y, Cao Q, Xiao Y, Wu Y, Ding L, Huang H, et al. The progress and future of the treatment of Candida albicans infections based on nanotechnology. Journal of Nanobiotechnology. 2024;22(1):568.
54. Sherry L, Ramage G, Kean R, Borman A, Johnson EM, Richardson MD, et al. Biofilm-forming capability of highly virulent, multidrug-resistant Candida auris. Emerging infectious diseases. 2017;23(2):328.
55. Ge S-H, Wan Z, Li J, Xu J, Li R-Y, Bai F-Y. Correlation between azole susceptibilities, genotypes, and ERG11 mutations in Candida albicans isolates associated with vulvovaginal candidiasis in China. Antimicrobial agents and chemotherapy. 2010;54(8):3126-31.
56. Mayer FL, Duncan W, and Hube B. Candida albicans pathogenicity mechanisms. Virulence. 2013;4(2):119-28.
57. Gonçalves SS, Souza ACR, Chowdhary A, Meis JF, Colombo AL. Epidemiology and molecular mechanisms of antifungal resistance in Candida and Aspergillus. Mycoses. 2016;59(4):198-219.

58. Popp C, Hampe IA, Hertlein T, Ohlsen K, Rogers PD, Morschhäuser J. Competitive fitness of fluconazole-resistant clinical Candida albicans strains. Antimicrobial Agents and Chemotherapy. 2017;61(7):10.1128/aac. 00584-17.

59. Gerstein AC, Berman J. Candida albicans genetic background influences mean and heterogeneity of drug responses and genome stability during evolution in fluconazole. Msphere. 2020;5(3):10.1128/msphere. 00480-20.

60. Chauhan V, Kumar A, Tripathi S, Jha M, Kumar N, Poluri KM, et al. An update on the pathogenesis and ethnopharmacological therapeutic approaches of vulvovaginal candidiasis. Discover Public Health. 2024;21(1):195.

61. Cavalheiro M, Teixeira MC. Candida biofilms: threats, challenges, and promising strategies. Frontiers in medicine. 2018;5:28.

62. Prigent G, Aït-Ammar N, Levesque E, Fekkar A, Costa J-M, El Anbassi S, et al. Echinocandin resistance in Candida species isolates from liver transplant recipients. Antimicrobial Agents and Chemotherapy. 2017;61(2):10.1128/aac. 01229-16.

63. Nash AK, Auchtung TA, Wong MC, Smith DP, Gesell JR, Ross MC, et al. The gut mycobiome of the Human Microbiome Project healthy cohort. Microbiome. 2017;5(1):153.

64. Li S, Su B, He Q-S, Wu H, Zhang T. Alterations in the oral microbiome in HIV infection: causes, effects and potential interventions. Chinese Medical Journal. 2021;134(23):2788-98.

65. Enitan SS, Ihongbe JC, Olumide A, Ochei JO, Itodo GE, Oluremi AS, et al. Candidiasis in HIV positive female adults on HAART in Ogun State, Nigeria: Prevalence, risk factors and antifungal susceptibility study. ARC Journal of AIDS. 2019;4(2):1-17.

66. Brown GD, Denning DW, Levitz SM. Tackling human fungal infections. American Association for the Advancement of Science; 2012. p. 647-.

67. Hill LM, Golin CE, Saidi F, Phanga T, Tseka J, Young A, et al. Understanding PrEP decision making among pregnant women in Lilongwe, Malawi: A mixed-methods study. Journal of the International AIDS Society. 2022;25(9):e26007.

68. miruka hn, Eric OO, Musyoki S, Awuor SO. Incidence, antifungal resistance properties, and virulence traits of candida species isolated from HIV/AIDS Patients from the hospital system in Kenya. Access Microbiology. 2023.

69. van de Wijgert JHHM, Borgdorff H, Verhelst R, Crucitti T, Francis S, Verstraelen H, et al. The Vaginal Microbiota: What Have We Learned after a Decade of Molecular Characterization? PLOS ONE. 2014;9(8):e105998.

70. Consuegra-Asprilla JM, Rodríguez-Echeverri C, Posada DH, Gómez BL, González Á. Patients with recurrent vulvovaginal candidiasis exhibit a decrease in both the fungicidal activity of neutrophils and the proliferation of peripheral blood mononuclear cells. Mycoses. 2024;67(4):e13720.

71. Demin KA, Refeld AG, Bogdanova AA, Prazdnova EV, Popov IV, Kutsevalova OY, et al. Mechanisms of candida resistance to antimycotics and promising ways to overcome it: the role of probiotics. Probiotics and antimicrobial proteins. 2021;13(4):926-48.
72. Fernandes CM, Dasilva D, Haranahalli K, McCarthy JB, Mallamo J, Ojima I, et al. The Future of Antifungal Drug Therapy: Novel Compounds and Targets. Antimicrobial Agents and Chemotherapy. 2021;65(2):10.1128/aac.01719-20.
73. Waikhom SD, Afeke I, Kwawu GS, Mbroh HK, Osei GY, Louis B, et al. Prevalence of vulvovaginal candidiasis among pregnant women in the Ho municipality, Ghana: species identification and antifungal susceptibility of Candida isolates. BMC Pregnancy and Childbirth. 2020;20(1):266.
74. Lukic A, Napoli A, Santino I, Bianchi P, Nobili F, Ciampittiello G, et al. Cervicovaginal bacteria and fungi in pregnant diabetic and non-diabetic women: a multicenter observational cohort study. European Review for Medical & Pharmacological Sciences. 2017;21(10).
75. Dermitzaki N, Baltogianni M, Tsekoura E, Giapros V. Invasive Candida Infections in Neonatal Intensive Care Units: Risk Factors and New Insights in Prevention. Pathogens. 2024;13(8):660.
76. Yao D, Tang X, Xue M, Zhu K, Li W, Chen Z, et al. The effects of vaginal infections on pregnancy outcomes: a retrospective cohort study in China. BMC Pregnancy and Childbirth. 2025;25(1):179.
77. Farr A, Effendy I, Frey Tirri B, Hof H, Mayser P, Petricevic L, et al. Guideline: Vulvovaginal candidosis (AWMF 015/072, level S2k). Mycoses. 2021;64(6):583-602.
78. Peters BM, Coleman BM, Willems HME, Barker KS, Aggor FEY, Cipolla E, et al. The Interleukin (IL) 17R/IL-22R Signaling Axis Is Dispensable for Vulvovaginal Candidiasis Regardless of Estrogen Status. J Infect Dis. 2020;221(9):1554-63.
79. Gedefie A, Shimeles G, Motbainor H, Kassanew B, Genet C. Vaginal colonization and vertical transmission of Candida species: prevalence and associated factors among pregnant women and their neonates at public health facilities of Northeast Ethiopia. BMC Pregnancy and Childbirth. 2025;25(1):22.
80. Ramírez-Lozada T, Espinosa-Hernández VM, Frías-De-León MG, Martínez-Herrera E. Update of Vulvovaginal Candidiasis in Pregnant and Non-pregnant Patients. Current Fungal Infection Reports. 2019;13(4):181-90.
81. Dekkers B, Veringa A, Marriott D, Boonstra J, Elst K, Doukas F, et al. Invasive Candidiasis in the Elderly: Considerations for Drug Therapy. Drugs & Aging. 2018;35:1-9.
82. Messina A, Mariani A, Brandolisio R, Tavella E, Germano C, Lipari G, et al. Candidiasis in Pregnancy: Relevant Aspects of the Pathology for the Mother and the Fetus and Therapeutic Strategies. Tropical Medicine and Infectious Disease. 2024;9(5):114.
83. Eskezia A, Teklemichael AM, Alemayehu T. The prevalence and risk factors of vaginal Candida species and group B Streptococcus colonization in pregnant women attending antenatal care at Hawassa

university comprehensive specialized hospital in Hawassa City, Southern Ethiopia. BMC Pregnancy and Childbirth. 2025;25(1):299.

84. Khani N, Abedi Soleimani R, Chadorshabi S, Moutab BP, Milani PG, Rad AH. Postbiotics as candidates in biofilm inhibition in food industries. Letters in Applied Microbiology. 2023;77(4).

85. Buniowska-Olejnik M, Urbański J, Mykhalevych A, Bieganowski P, Znamirowska-Piotrowska A, Kačániová M, et al. The influence of curcumin additives on the viability of probiotic bacteria, antibacterial activity against pathogenic microorganisms, and quality indicators of low-fat yogurt. Frontiers in Nutrition. 2023;Volume 10 - 2023.

86. Das R, Kotra K, Singh P, Loh B, Leptihn S, Bajpai U. Alternative Treatment Strategies for Secondary Bacterial and Fungal Infections Associated with COVID-19. Infectious Diseases and Therapy. 2022;11(1):53-78.

87. Fisher MC, Alastruey-Izquierdo A, Berman J, Bicanic T, Bignell EM, Bowyer P, et al. Tackling the emerging threat of antifungal resistance to human health. Nature reviews microbiology. 2022;20(9):557-71.

88. Iyer KR, Revie NM, Fu C, Robbins N, Cowen LE. Treatment strategies for cryptococcal infection: challenges, advances and future outlook. Nature Reviews Microbiology. 2021;19(7):454-66.

89. Cavassin FB, Baú-Carneiro JL, Vilas-Boas RR, Queiroz-Telles F. Sixty years of amphotericin B: an overview of the main antifungal agent used to treat invasive fungal infections. Infectious diseases and therapy. 2021;10(1):115-47.

90. Wall G, Lopez-Ribot JL. Screening repurposing libraries for identification of drugs with novel antifungal activity. Antimicrobial Agents and Chemotherapy. 2020;64(9):10.1128/aac. 00924-20.

91. Haddaji N, Bahloul B, Bahia W, Bechambi O, Mahdhi A. Development of Nanotechnology-Based Drug Delivery Systems for Controlling Clinical Multidrug-Resistant Staphylococcus aureus and Escherichia coli Associated with Aerobic Vaginitis. Pharmaceutics. 2023;15(8):2133.

92. Naicker D, Govender R, Abbai NS. Busting the Resistance: Antimicrobial Activity of Plant-Infused Nanoemulsions against Neisseria gonorrhoeae. International Journal of Microbiology. 2024;2024(1):7084347.

93. Muthamil S, Prasath KG, Priya A, Precilla P, Pandian SK. Global proteomic analysis deciphers the mechanism of action of plant derived oleic acid against Candida albicans virulence and biofilm formation. Scientific Reports. 2020;10(1):5113.

94. Gizaw A, Marami LM, Teshome I, Sarba EJ, Admasu P, Babele DA, et al. Phytochemical screening and in vitro antifungal activity of selected medicinal plants against Candida albicans and Aspergillus niger in West Shewa Zone, Ethiopia. Advances in Pharmacological and Pharmaceutical Sciences. 2022;2022(1):3299146.

95. Lin Y-T, Tsai W-C, Lu H-Y, Fang S-Y, Chan H-W, Huang C-H. Enhancing Therapeutic Efficacy of Cinnamon Essential Oil by Nanoemulsification for Intravaginal Treatment of Candida Vaginitis. International Journal of Nanomedicine. 2024:4941-56.

96. Lemos AS, Florêncio JR, Pinto NC, Campos LM, Silva TP, Grazul RM, et al. Antifungal activity of the natural coumarin scopoletin against planktonic cells and biofilms from a multidrug-resistant Candida tropicalis strain. Frontiers in Microbiology. 2020;11:1525.
97. Zielińska S, Wójciak-Kosior M, Dziągwa-Becker M, Gleńsk M, Sowa I, Fijałkowski K, et al. The Activity of Isoquinoline Alkaloids and Extracts from Chelidonium majus against Pathogenic Bacteria and Candida sp. Toxins. 2019;11(7):406.
98. Merenkova S, Zinina O, Potoroko I. Fermented Plant Beverages Stabilized with Microemulsion: Confirmation of Probiotic Properties and Antioxidant Activity. Fermentation. 2022;8(12):723.
99. Abdel-Nasser M, Abdel-Maksoud G, Eid AM, Hassan SE-D, Abdel-Nasser A, Alharbi M, et al. Antifungal Activity of Cell-Free Filtrate of Probiotic Bacteria Lactobacillus rhamnosus ATCC-7469 against Fungal Strains Isolated from a Historical Manuscript. Microorganisms. 2023;11(5):1104.
100. Ribeiro FC, Rossoni RD, de Barros PP, Santos JD, Fugisaki LRO, Leão MPV, et al. Action mechanisms of probiotics on Candida spp. and candidiasis prevention: an update. Journal of Applied Microbiology. 2020;129(2):175-85.
101. Jang SJ, Lee K, Kwon B, You HJ, Ko G. Vaginal lactobacilli inhibit growth and hyphae formation of Candida albicans. Scientific Reports. 2019;9(1):8121.
102. Martinez RCR, Franceschini SA, Patta MC, Quintana SM, Candido RC, Ferreira JC, et al. Improved treatment of vulvovaginal candidiasis with fluconazole plus probiotic Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14. Letters in Applied Microbiology. 2009;48(3):269-74.
103. Radwan IT, El-Sherbiny IM, Metwally NH. Synergistic and potential antifungal properties of tailored, one pot multicomponent monoterpenes co-delivered with fluconazole encapsulated nanostructure lipid carrier. Scientific Reports. 2024;14(1):14382.
104. Mohamed F, Ibrahim GA, Sharaf OM. Attempts to Improve Antimicrobial Efficiency by Mixed-Lactobacillus Extracts as Crude or Nano-formulated Against Pathogenic and Food Spoilage Bacteria, Molds and Yeasts. 2021.
105. Cleveland AA, Harrison LH, Farley MM, Hollick R, Stein B, Chiller TM, et al. Declining Incidence of Candidemia and the Shifting Epidemiology of Candida Resistance in Two US Metropolitan Areas, 2008–2013: Results from Population-Based Surveillance. PLOS ONE. 2015;10(3):e0120452.
106. Nguyen RH, Reese RL, Harlow BL. Differences in pain subtypes between Hispanic and non-Hispanic white women with chronic vulvar pain. Journal of women's health. 2015;24(2):144-50.
107. Thomas TL, Yarandi HN, Dalmida SG, Frados A, Klienert K. Cross-cultural differences and sexual risk behavior of emerging adults. Journal of Transcultural Nursing. 2015;26(1):64-72.
108. Thomas-White K, Navarro P, Wever F, King L, Dillard LR, Krapf J. Psychosocial impact of recurrent urogenital infections: a review. Women's Health. 2023;19:17455057231216537.

109. Ostrosky-Zeichner L, Shoham S, Vazquez J, Reboli A, Betts R, Barron MA, et al. MSG-01: A Randomized, Double-Blind, Placebo-Controlled Trial of Caspofungin Prophylaxis Followed by Preemptive Therapy for Invasive Candidiasis in High-Risk Adults in the Critical Care Setting. Clinical Infectious Diseases. 2014;58(9):1219-26.
110. Weir E, Allison C, Baron-Cohen S. The sexual health, orientation, and activity of autistic adolescents and adults. Autism Research. 2021;14(11):2342-54.
111. Miyazima TY, Ishikawa KH, Mayer MPA, Saad SMI, Nakamae AEM. Cheese supplemented with probiotics reduced the Candida levels in denture wearers—RCT. Oral Diseases. 2017;23(7):919-25.
112. Uppuluri A, Zarbin MA, Budoff G, Bhagat N. Risk Factors for Endogenous Endophthalmitis in Hospitalized Patients with Candida Fungemia. Ophthalmology Retina. 2021;5(7):687-95.
113. Ranjit E, Raghubanshi BR, Maskey S, Parajuli P. Prevalence of Bacterial Vaginosis and Its Association with Risk Factors among Nonpregnant Women: A Hospital Based Study. International Journal of Microbiology. 2018;2018(1):8349601.
114. Rotem R, Fishman B, Daniel S, Koren G, Lunenfeld E, Levy A. Risk of major congenital malformations following first-trimester exposure to vaginal azoles used for treating vulvovaginal candidiasis: a population-based retrospective cohort study. BJOG: An International Journal of Obstetrics & Gynaecology. 2018;125(12):1550-6.
115. Andes DR, Safdar N, Baddley JW, Alexander B, Brumble L, Freifeld A, et al. The epidemiology and outcomes of invasive Candida infections among organ transplant recipients in the United States: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). Transplant Infectious Disease. 2016;18(6):921-31.
116. Yokoyama H, Nagao A, Watanabe S, Honjo J. Incidence and risk of vaginal candidiasis associated with sodium–glucose cotransporter 2 inhibitors in real-world practice for women with type 2 diabetes. Journal of Diabetes Investigation. 2019;10(2):439-45.
117. Jenks JD, Aneke CI, Al-Obaidi MM, Egger M, Garcia L, Gaines T, et al. Race and ethnicity: Risk factors for fungal infections? PLOS Pathogens. 2023;19(1):e1011025.
118. Africa CWJ, Turton M. Oral Health Status and Treatment Needs of Pregnant Women Attending Antenatal Clinics in KwaZulu-Natal, South Africa. International Journal of Dentistry. 2019;2019(1):5475973.
119. Zhang M-r, Zhao F, Wang S, Lv S, Mou Y, Yao C-l, et al. Molecular mechanism of azoles resistant Candida albicans in a patient with chronic mucocutaneous candidiasis. BMC Infectious Diseases. 2020;20:1-6.
120. Nobile CJ, Fox EP, Hartooni N, Mitchell KF, Hnisz D, Andes DR, et al. A histone deacetylase complex mediates biofilm dispersal and drug resistance in Candida albicans. MBio. 2014;5(3):10.1128/mbio. 01201-14.

121. Bidaud A, Botterel F, Chowdhary A, Dannaoui E. In vitro antifungal combination of flucytosine with amphotericin B, voriconazole, or micafungin against Candida auris shows no antagonism. Antimicrobial agents and chemotherapy. 2019;63(12):10.1128/aac. 01393-19.
122. Payne MS, Ireland DJ, Watts R, Nathan EA, Furfaro LL, Kemp MW, et al. Ureaplasma parvum genotype, combined vaginal colonisation with Candida albicans, and spontaneous preterm birth in an Australian cohort of pregnant women. BMC Pregnancy and Childbirth. 2016;16(1):312.
123. Schuster HJ, de Jonghe BA, Limpens J, Budding AE, Painter RC. Asymptomatic vaginal Candida colonization and adverse pregnancy outcomes including preterm birth: a systematic review and meta-analysis. American Journal of Obstetrics & Gynecology MFM. 2020;2(3):100163.

BRIDGE

Chapter 1 provided an overview of the global and local landscape of *C. albicans* infections, highlighting the rising burden of VVC, emerging antifungal resistance, and the complex interplay between host factors, strain diversity, and microbial pathogenesis. The chapter also emphasized the need for regionally relevant data that can inform diagnosis, treatment, and surveillance efforts in South Africa. A key gap identified in the literature is the limited understanding of *C. albicans* strain variation at the genotypic level, and how such diversity may influence antifungal susceptibility and clinical outcomes. In response to these gaps, Chapter 2 presents the first experimental component of this thesis. This chapter characterises the *C. albicans* isolates recovered from South African women through ABC genotyping and antifungal susceptibility testing. By examining the relationship between genotype distribution and resistance patterns, this chapter provides foundational insights into the strain dynamics circulating in this population and establishes an essential context for the molecular investigations that follow.

CHAPTER 2

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Correlation between genotypes and antifungal susceptibility profiles of *Candida* isolates from pregnant and non-pregnant women in South Africa

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Abstract

Vulvovaginal candidiasis (VVC) is a common vaginal infection, affecting as many as 75% of women of reproductive age at some point in their lives. The leading cause of VVC is *Candida albicans* (*C. albicans*). This study investigates the correlation between genotypes and antifungal susceptibility profiles of *Candida* isolates collected from pregnant and non-pregnant South African women. A total of 72 *Candida* isolates were identified using the Applied Biosystems TaqMan® Assay and confirmed via germ tube tests and polymerase chain reaction (PCR). All isolates (100%) were identified as *C. albicans*. ABC genotyping revealed that 62.5% of isolates were genotype A, 26.4% genotype B, and 11.1% genotype C. Antifungal susceptibility testing using the Sensititre™ YeastOne™ YO10 AST Plate assessed minimum inhibitory concentrations (MICs) for anidulafungin, caspofungin, fluconazole, micafungin, and voriconazole. Fluconazole showed the highest resistance rate (13.9%), while 86.1% of isolates remained susceptible. Genotype A predominated among isolates resistant to anidulafungin, fluconazole, micafungin, and voriconazole. All caspofungin-resistant isolates were genotype C. Genotype B exhibited no resistance to any antifungals tested, indicating the lowest virulence among the genotypes. These findings suggest that genotypes A and C have higher resistance profiles, emphasizing the need for routine VVC screening and resistance surveillance to inform effective management of *Candida* infections.

Keywords: vulvovaginal candidiasis, drug resistance, polymerase chain reaction, antifungal agents, pregnancy complications

Introduction

Vulvovaginal candidiasis (VVC) is a prevalent vaginal infection, impacting up to 75% of women of reproductive age at least once during their lifetime.[1,2] The primary organism responsible for VVC is *Candida albicans* (*C. albicans*), which is responsible for 70% to 90% of VVC cases.[3] Previous research has highlighted a high prevalence of *Candida* infections in Africa and the Middle East, with reported rates of 55.18% and 76.92%, respectively.[4] In sub-Saharan and central Africa, the infection rate for *C. albicans* was found to be 22.74%, while the South African region had a slightly lower rate of 22.44%. Candidiasis ranks as the fifth most common life-threatening fungal infection, with an estimated mortality rate of 40%.[5] Symptoms of VVC often include redness around the genital area, inflammation of the genital tract, itching, and a thick white discharge.[6]

The identification of *Candida* species from culture-positive women is crucial for determining the species responsible for the infection, as well as assessing antimicrobial susceptibility and resistance mechanisms.[7] Accurate identification of *Candida* species is important, as their responses to antifungal drugs can differ, which helps ensure effective therapy and reduces the risk of treatment failure. Various molecular techniques, such as Southern blotting hybridization, multilocus sequence typing (MLST), and deoxyribonucleic acid (DNA) microsatellite typing, have been employed for the genotyping of *Candida* isolates. These genotyping methods classify strains into clades, which are groups derived from a common ancestor. Studies have shown that the distribution of these clades is influenced by geographical factors and antifungal resistance patterns, with certain clades being linked to specific resistance profiles.[8] The ABC genotyping method is commonly used for *C. albicans*, where polymerase chain reaction (PCR) amplification of the *25S ribosomal deoxyribonucleic acid (rDNA)* allows for the classification of isolates into genotypes A: 450 bp (base pair), B: 840 bp, C: 450 bp and 840 bp, and D: 1080 bp.[7] A study by Jafarian and colleagues reported genotype A at a prevalence of 57.9%, genotype B at 31.6%, and genotype C at 10.5% among 933 patients, of whom 23 were confirmed to have *Candida* infections.[9]

Antifungal drug resistance is a significant contributor to the treatment failure of *Candida* infections.[10] Treatment options for candidiasis remain limited, even though various antifungal drugs exist. Based on their mechanisms of action, antifungal drugs used to manage candidiasis are divided into four classes: (1) Disruption of cell membrane sterol (polyenes, such as amphotericin B and nystatin); (2) Inhibition of the ergosterol biosynthesis pathway (azoles, including fluconazole, voriconazole, posaconazole, and ravuconazole); (3) Inhibition of DNA or ribonucleic acid synthesis (flucytosine); (4) Inhibition of glucan synthesis (echinocandins, such as caspofungin, micafungin, and anidulafungin).[11] Fluconazole remains the most frequently used azole for both the prevention and treatment of *Candida* infections. However, prolonged use of this drug can lead to the development of resistance among *Candida* species reducing its effectiveness, this has been supported by previous research where resistance to fluconazole among *Candida* species has become a growing concern within healthcare systems.[12]

Although global research has advanced in linking *C. albicans* genotypes with antifungal susceptibility profiles, there is limited international data and a lack of South African data. Therefore, addressing this gap is critical, especially considering the high burden of candidiasis in the region and the potential public health consequences of increasing treatment resistance. This study aims to explore the correlation between genotypes and antifungal susceptibility profiles of anidulafungin, caspofungin, fluconazole, micafungin and voriconazole among *C. albicans* isolates from pregnant and non-pregnant South African women, providing a novel insight into the dynamics of drug resistance. By linking the genotypes to antifungal susceptibility profiles, this research contributes to a better understanding of resistance mechanisms, potentially creating more effective therapeutic strategies and enhancing regional treatment guidelines.

Methodology

Study setting and population derived from the parent study

This study was a sub-study of a broader research project, approved by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (UKZN) under reference number (BREC/00003674/2021), which focused on diagnosing vaginitis and vaginosis pathogens in women. In the parent study, 150 women were recruited from Victoria Mxenge Hospital in Durban, KwaZulu-Natal, South Africa. Participants in the main study were 18 years or older, provided written informed consent, and agreed to self-collect vaginal swabs, following sample collection instructions from the research team. Data on sexual behaviour, clinical history, and socio-demographic details were gathered from each participant through a structured questionnaire administered by the study team. The recruitment period for the study population spanned from January to August 2022.

Ethical approval for the sub-study

Approval for this sub-study was granted by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (reference number BREC/00005995/2023).

Laboratory procedures

Sample collection and processing of vaginal swabs from the parent study

Following sample collection, the swabs for *Candida* detection were placed in a 15 millilitre (ml) tube with Cary-Blair Transport Media (Neogen, United States) and transported to the Clinical Medicine Laboratory at UKZN for culture analysis. At the laboratory, the swabs were streaked onto Sabouraud dextrose agar (SDA) plates containing chloramphenicol (Neogen, United States) and incubated at 35°C

for 48 hours. After incubation, a total of 72 isolates showed positive *Candida* cultures, with 31 isolates derived from pregnant women and 41 from non-pregnant women. These cultures were stored at -80°C for future use.

Retrieval from storage for the sub-study

The 72 stored *Candida* cultures were retrieved from storage and sub-cultured onto SDA plates containing chloramphenicol (Neogen, United States) and incubated at 35°C for 48 hours.

Confirmatory assays for the isolates

The germ tube test

The germ tube test was conducted to distinguish *C. albicans* from other *Candida* species such as *C. krusei*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*. For this test, 0.5 ml of fetal calf serum (ThermoFisher Scientific, United States), was added to microfuge tubes, and a single colony from the SDA plate culture was mixed into the serum. The tubes were incubated at 37°C for 2 to 3 hours. After incubation, wet mount microscopy was used to observe germ tube formation. A positive result for *C. albicans* was indicated by short hyphal (filamentous) extensions emerging laterally from yeast cells without constriction at their origin. Samples without hyphal extensions or with constricted short hyphae at their origin were categorized as negative or as other yeast species.[13]

DNA extraction

DNA extraction from *Candida* cultures were carried out using the PureLink Microbiome Kit (ThermoFisher Scientific, United States) following the manufacturer's protocol. The extracted DNA was stored at -20°C. A Nanodrop Spectrophotometer (ThermoFisher Scientific, United States) was used to measure the concentration and purity of the DNA.

Confirmation of Candida isolates by real-time PCR

The identity of *Candida* isolates was verified using the Applied Biosystems TaqMan® Assays (ThermoFisher Scientific, United States) with commercially available primers and probes targeting *C. albicans*, *C. lusitaniae*, *C. dubliniensis*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis,* and *C. krusei*. The assays were conducted on the QuantStudio 5 Real-time PCR detection system (ThermoFisher Scientific, United States). Each PCR reaction had a final volume of 5 microliters (µl), consisting of 0.25 µl Fluorescein Amidite-labelled probe/primer mix, 1.25 µl Fast Start 4x probe master mix (Thermofisher, Part No. 4444434), 2 µl template DNA, and 1.5 µl nuclease-free water. A positive control (TaqMan™ Vaginal Microbiota Extraction Control; cat no. A32039) and a non-template control were included. Amplification involved an initial step at 95°C for 30 seconds, followed by 45 cycles of denaturation at 95°C for 3 seconds and annealing at 60°C for 30 seconds. Fluorescent signals from amplified products

were detected at the end of the annealing phase, and the QuantStudio 5 system software automatically generated raw fluorescent data, including threshold cycle (C_t) mean values.

Genotyping of the C. albicans isolates

The isolates were typed using the ABC genotyping method. The *25S rDNA* gene was amplified from the previously extracted DNA using primers CA-INT-L (5′-ATA AGG GAA GTC GGC AAA ATA GAT CCG TAA-3′) and CA-INT-R (5′-CCT TGG CTG TGG TTT CGC TAG ATA GTA GAT-3′). The PCR master mix included 200 nanometres (nm) of each primer, 12.5 µl of DreamTaq (2x) Master Mix (ThermoFisher Scientific, United States), 9.5 µl of nuclease-free water, and 2 µl of template DNA. The PCR tubes were then placed in a BioRad thermal cycler, and cycling conditions were set to 95°C for 2 minutes, followed by 35 cycles of 95°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 7 minutes. PCR products were electrophoresed on a 1% agarose gel and visualized using an ultraviolet transilluminator. Based on the yielded band sizes, the *C. albicans* isolates were classified as genotype A (450 bp), genotype B (840 bp), genotype C (450 bp and 840 bp), and genotype D (1080 bp).[14]

Antifungal susceptibility assay for C. albicans

Susceptibility testing was conducted using the Sensititre™ YeastOne™ YO10 AST Plate (ThermoFisher Scientific, United States) to assess the minimum inhibitory concentrations (MICs) of *C. albicans* isolates against anidulafungin, caspofungin, fluconazole, micafungin, and voriconazole. An inoculum of *C. albicans* for each isolate was prepared to match a 0.5 McFarland standard. From this suspension, 20 µl was added to the Sensititre YeastOne Broth, followed by the addition of 100 µl of the inoculum into the microtiter plates. The plates were sealed and incubated at 35°C for 24 to 25 hours. The *C. albicans* American Type Culture Collection (ATCC) 10231-strain served as a control strain, while untreated cultures of each isolate were included as growth controls. All experiments were conducted in triplicate. Table 1 displays the MIC breakpoints, as per the Clinical and Laboratory Standards Institute (CLSI) guidelines.

Table 1: MIC breakpoints as per CLSI guidelines.

Antifungal	Susceptible (µg/ml)	SDD (µg/ml)	Resistant (µg/ml)
Anidulafungin	≤ 0.25	0.5	≥ 1
Caspofungin	≤ 0.25	0.5	≥ 1
Fluconazole	≤ 2	4	≥ 8
Micafungin	≤ 0.25	0.5	≥ 1
Voriconazole	≤ 0.12	0.25 - 0.5	≥ 1

MIC - minimum inhibitory concentrations, SDD - susceptible-dose-dependent

Results

Confirmatory assays for the obtained isolates

All isolates (72/72; 100%) were confirmed as *C. albicans* based on the germ tube test (Figure 1). Further confirmation was achieved through the quantitative PCR assay using primers and probes specific to *Candida* species. Table 2 presents the amplification results from the TaqMan assay using these specific probes and primers, indicating that all samples yielded positive amplification. The positive and negative controls also produced the expected results.

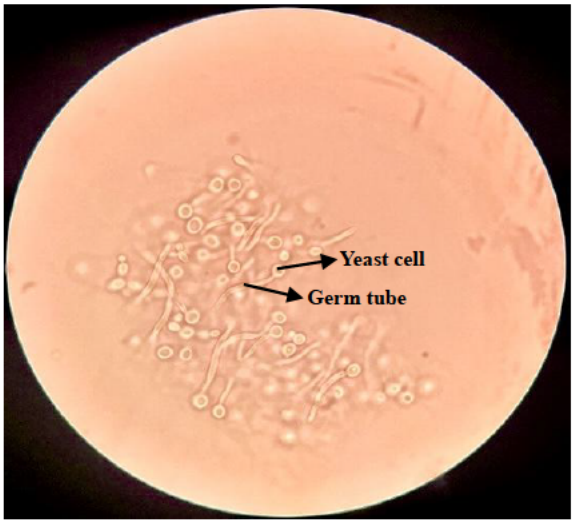

Figure 1: A microscope slide illustrating the results of the germ tube test, which was examined using oil immersion at 100X magnification.

Table 2: Results from the TaqMan assay utilizing primers and probes specific to *Candida* species, indicating the amplification outcomes for the tested samples.

Isolate name	TaqMan assay result (C_t value)
ZMO1	Positive (15.9)
ZMO10	Positive (30.8)
ZMO11	Positive (17.5)
ZMO12	Positive (16.7)
ZMO14	Positive (19.8)
ZMO17	Positive (20.2)
ZMO18	Positive (26.3)
ZMO20	Positive (32.9)
ZMO21	Positive (14.0)
ZMO23	Positive (12.3)
ZMO25	Positive (15.3)
ZMO27	Positive (14.5)
ZMO28	Positive (29.1)
ZMO29	Positive (30.9)
ZMO30	Positive (15.3)
ZMO32	Positive (28.6)
ZMO34	Positive (17.8)
ZMO35	Positive (14.9)
ZMO37	Positive (19.6)
ZMO40	Positive (33.1)
ZMO41	Positive (17)
ZMO42	Positive (31.3)
ZMO43	Positive (17.7)
ZMO44	Positive (24.9)
ZMO47	Positive (16.2)
ZMO53	Positive (28.6)
ZMO54	Positive (17.4)
ZMO56	Positive (17.8)
ZMO58	Positive (17.9)
ZMO59	Positive (18.9)
ZMO60	Positive (15.8)
ZMO62	Positive (24.7)
ZMO63	Positive (16.8)

ZMO65	Positive (26.6)
ZMO67	Positive (19.3)
ZMO68	Positive (29.6)
ZMO69	Positive (25.1)
ZMO71	Positive (12.4)
ZMO72	Positive (21.5)
ZMO75	Positive (18.5)
ZMO77	Positive (17)
ZMO79	Positive (27.9)
ZMO80	Positive (19.2)
ZMO81	Positive (22)
ZMO82	Positive (29.0)
ZMO83	Positive (16.5)
ZMO84	Positive (20.2)
ZMO85	Positive (19.1)
ZMO86	Positive (15.4)
ZMO87	Positive (18.8)
ZMO88	Positive (16.9)
ZMO89	Positive (16.4)
ZMO91	Positive (14.6)
ZMO94	Positive (14.9)
ZMO95	Positive (18.8)
ZMO96	Positive (15.1)
ZMO97	Positive (16.3)
ZMO98	Positive (17.9)
ZMO99	Positive (28.3)
ZMO102	Positive (16.0)
ZMO103	Positive (15.2)
ZMO107	Positive (27.2)
ZMO110	Positive (31.1)
ZMO119	Positive (18.8)
ZMO128	Positive (15.9)
ZMO132	Positive (15.5)
ZMO135	Positive (32.2)
ZMO141	Positive (14.9)
ZMO142	Positive (31.1)

ZMO145	Positive (31.7)
ZMO146	Positive (13.6)
ZMO147	Positive (13.7)

C_t - cycle threshold

Genotyping analysis

All 72 isolates (100%) produced positive PCR results (Figures 3, 4, 5, 6, 7 and 8). Figure 2 shows that most of the isolates (45/72; 62.5%) exhibited a 450 bp band, which was classified as genotype A. Nineteen isolates (19/72; 26.4%) displayed a band size of 840 bp and were assigned as genotype B. Additionally, eight isolates (8/72; 11.1%) yielded two band sizes of 450 bp and 840 bp, corresponding to genotype C. No isolates were classified as genotype D since the 1080 bp band was not detected. A detailed summary of the assigned genotypes is shown in Table 3.

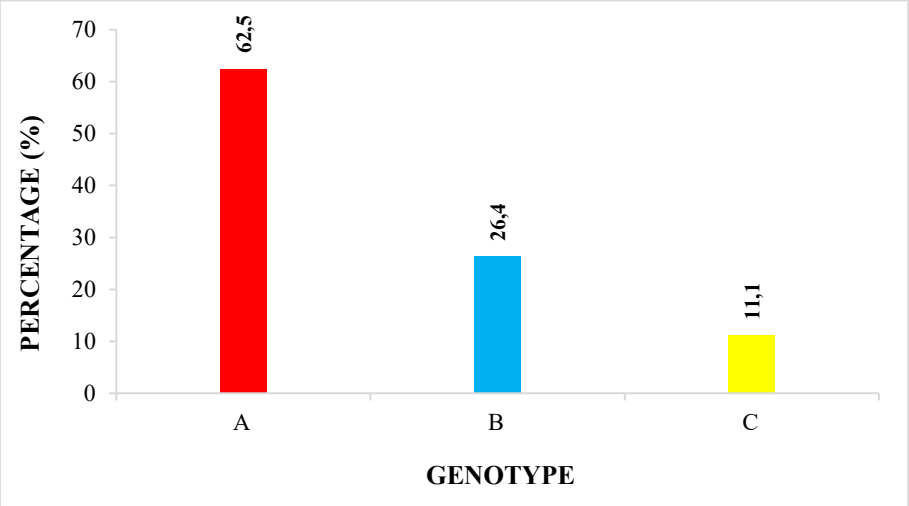

Figure 2: The percentages of *C. albicans* genotypes.

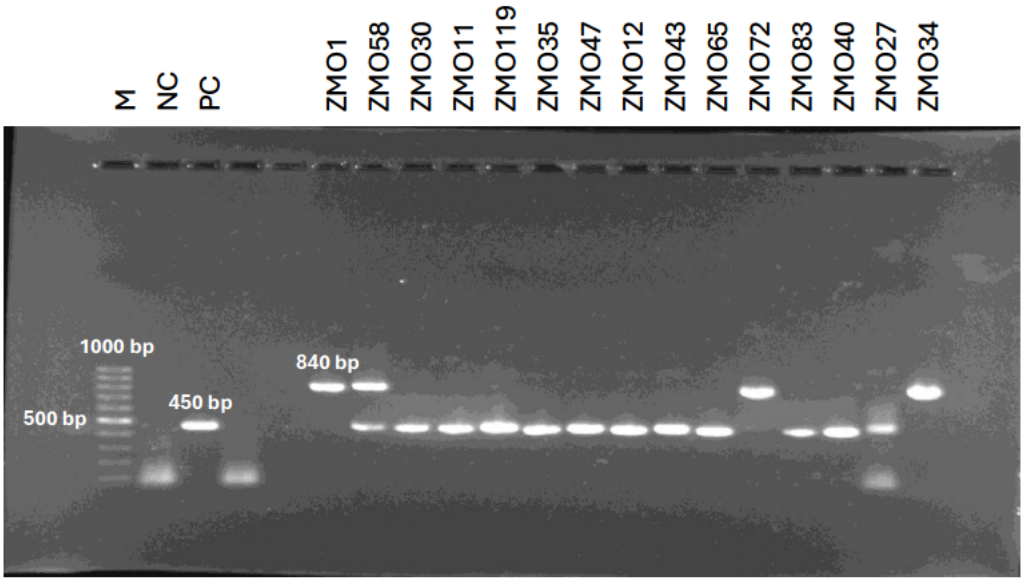

Figure 3: An agarose gel displaying positive amplicons generated for *C. albicans* isolates is shown, with observed band sizes of 450 bp, 840 bp, and a combination of both 450 bp and 840 bp. M represents the 100 bp DNA molecular ladder (ThermoFisher Scientific), NC indicates the negative control (no template DNA added), PC denotes the positive control (*C. albicans* ATCC 10231-strain), along with 15 clinical isolates of *C. albicans*.

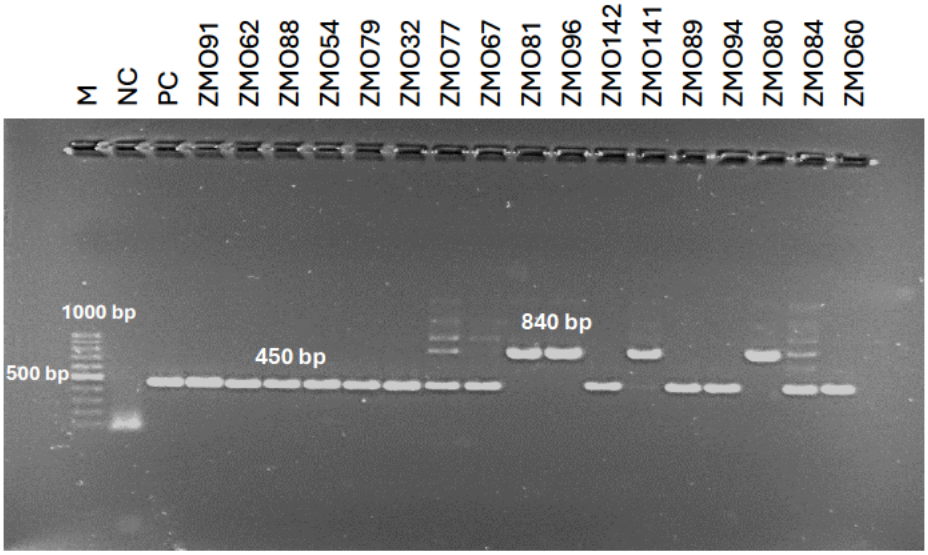

Figure 4: An agarose gel illustrating positive amplicons generated for *C. albicans* isolates is presented, with observed band sizes of 450 bp and 840 bp. M indicates the 100 bp DNA molecular ladder (ThermoFisher Scientific), NC represents the negative control (no template DNA added), PC denotes the positive control (*C. albicans* ATCC 10231-strain), and the gel includes 17 clinical isolates of *C. albicans*.

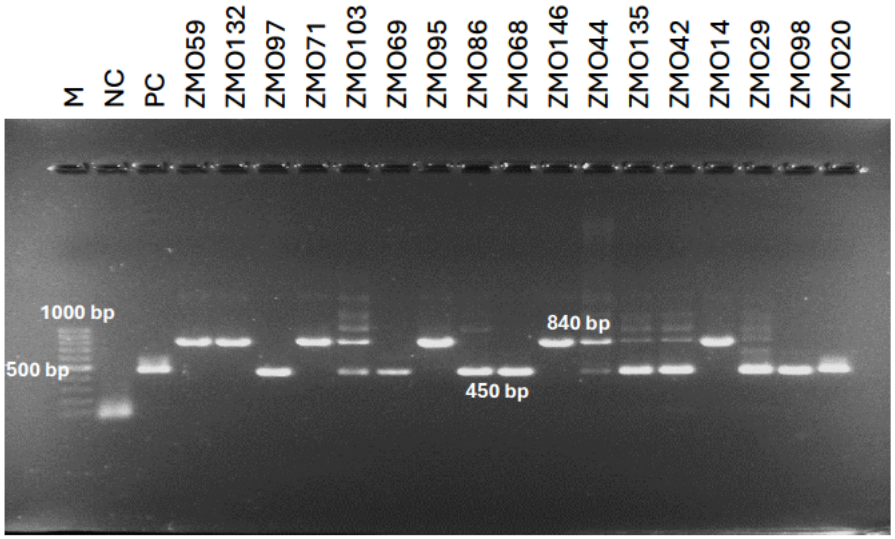

Figure 5: An agarose gel is presented, displaying positive amplicons generated for *C. albicans* isolates. The observed band sizes include 450 bp, 840 bp, and a combination of both 450 bp and 840 bp bands. M indicates the 100 bp DNA molecular ladder (ThermoFisher Scientific), NC represents the negative control (no template DNA added), PC denotes the positive control (*C. albicans* ATCC 10231-strain), along with 17 clinical isolates of *C. albicans*.

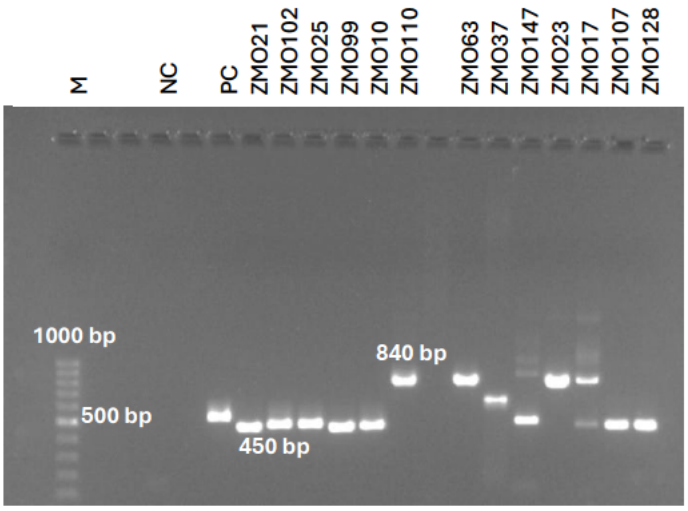

Figure 6: An agarose gel is shown, displaying positive amplicons generated for *C. albicans* isolates. The observed band sizes include 450 bp, 840 bp, and a combination of both 450 bp and 840 bp bands. M denotes the 100 bp DNA molecular ladder (ThermoFisher Scientific), NC indicates the negative control (no template DNA added), PC represents the positive control (*C. albicans* ATCC 10231-strain), and the gel includes 13 clinical isolates of *C. albicans*.

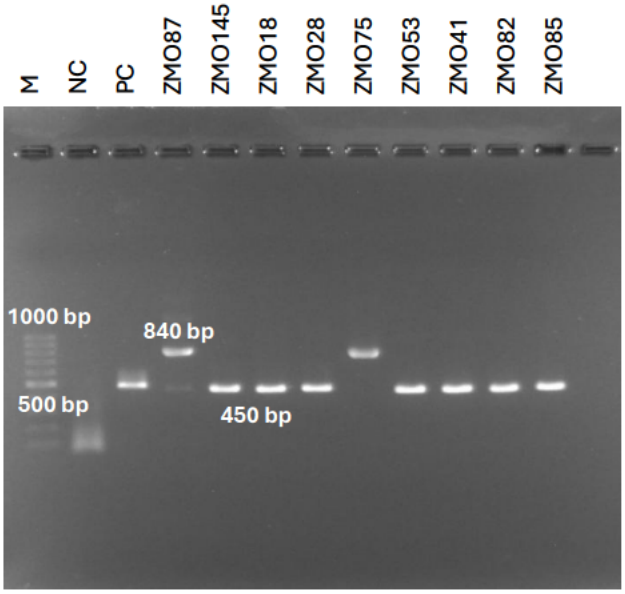

Figure 7: An agarose gel is presented, showing positive amplicons generated for *C. albicans* isolates. The observed band sizes include 450 bp, 840 bp, and a combination of both 450 bp and 840 bp bands. M indicates the 100 bp DNA molecular ladder (ThermoFisher Scientific), NC represents the negative control (no template DNA added), PC denotes the positive control (*C. albicans* ATCC 10231-strain), and the gel contains nine clinical isolates of *C. albicans*.

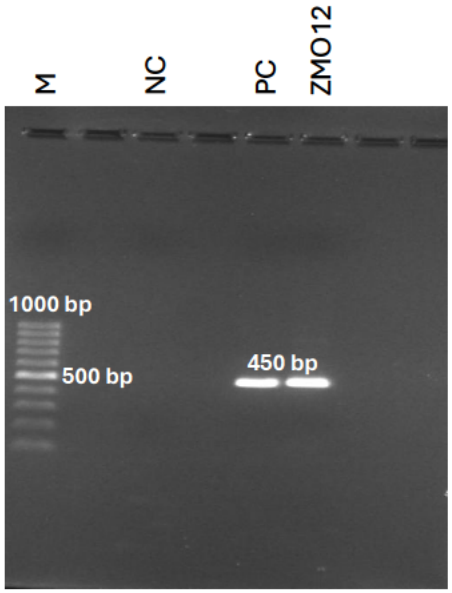

Figure 8: An agarose gel is displayed, showing positive amplicons generated for *C. albicans* isolates, with observed band sizes of 450 bp. M denotes the 100 bp DNA molecular ladder (ThermoFisher Scientific), NC represents the negative control (no template DNA added), PC indicates the positive control (*C. albicans* ATCC 10231-strain), and the gel includes one clinical isolate of *C. albicans*.

Table 3: Assignment of genotypes for individual isolates based on the banding patterns obtained.

Isolate name	PCR product size (bp)	Genotype
ZMO1	840	B
ZMO10	450	A
ZMO11	450	A
ZMO12	450	A
ZMO14	840	B
ZMO17	450 & 840	C
ZMO18	450	A
ZMO20	450	A
ZMO21	450	A
ZMO23	840	B
ZMO25	450	A
ZMO27	450	A
ZMO28	450	A
ZMO29	450	A
ZMO30	450	A
ZMO32	450	A
ZMO34	840	B
ZMO35	450	A
ZMO37	840	B
ZMO40	450	A
ZMO41	450	A
ZMO42	450 & 840	C
ZMO43	450	A
ZMO44	450 & 840	C
ZMO47	450	A
ZMO53	450	A
ZMO54	450	A
ZMO56	450	A
ZMO58	450 & 840	C
ZMO59	840	B
ZMO60	450	A
ZMO62	450	A
ZMO63	840	B
ZMO65	450	A

ZMO67	450	A
ZMO68	450	A
ZMO69	450	A
ZMO71	840	B
ZMO72	840	B
ZMO75	840	B
ZMO77	450 & 840	C
ZMO79	450	A
ZMO80	840	B
ZMO81	840	B
ZMO82	450	A
ZMO83	450	A
ZMO84	450 & 840	C
ZMO85	450	A
ZMO86	450	A
ZMO87	840	B
ZMO88	450	A
ZMO89	450	A
ZMO91	450	A
ZMO94	450	A
ZMO95	840	B
ZMO96	840	B
ZMO97	450	A
ZMO98	450	A
ZMO99	450	A
ZMO102	450	A
ZMO103	450 & 840	C
ZMO107	450	A
ZMO110	840	B
ZMO119	450	A
ZMO128	450	A
ZMO132	840	B
ZMO135	450 & 840	C
ZMO141	840	B
ZMO142	450	A
ZMO145	450	A

ZMO146	840	B
ZMO147	450	A

PCR - polymerase chain reaction, bp - base pair

Antifungal susceptibility assays

For anidulafungin, 91.7% of the 72 isolates tested (66/72) were susceptible, exhibiting MICs ≤ 0.25 µg/ml. Additionally, 1.4% of the isolates (1/72) were classified as susceptible-dose dependent (SDD) to anidulafungin, with a 0.5 µg/ml MIC. Meanwhile, 6.9% of the isolates (5/72) showed resistance to anidulafungin, displaying MICs ≥ 1 µg/ml (Supplementary Table 1). For caspofungin, 94.4% of the 72 isolates tested (68/72) were susceptible, exhibiting MICs ≤ 0.25 µg/ml. Additionally, 2.8% of the isolates (2/72) were classified as SDD to caspofungin, with a 0.5 µg/ml MIC. Similarly, 2.8% of the isolates (2/72) were resistant to caspofungin, displaying MICs ≥ 1 µg/ml (Supplementary Table 2). For fluconazole, 86.1% of the 72 isolates tested (62/72) were susceptible, exhibiting MICs ≤ 2 µg/ml. Conversely, 13.9% of the isolates (10/72) were found to be resistant to fluconazole, with MICs ≥ 8 µg/ml (Supplementary Table 3).

For micafungin, 90.3% of the 72 isolates tested (65/72) were susceptible, exhibiting MICs ≤ 0.25 µg/ml. Additionally, 5.5% of the isolates (4/72) were classified as SDD to micafungin, with a 0.5 µg/ml MIC. Furthermore, 4.2% of the isolates (3/72) were resistant to micafungin, displaying MICs ≥ 1 µg/ml (Supplementary Table 4). For voriconazole, 86% of the 72 isolates tested (62/72) were susceptible, exhibiting MICs ≤ 0.12 µg/ml. Additionally, 7% of the isolates (5/72) were classified as SDD to voriconazole, with MICs ranging from 0.25 µg/ml to 0.5 µg/ml. Furthermore, another 7% of the isolates (5/72) were resistant to voriconazole, displaying MICs ≥ 1 µg/ml (Supplementary Table 5). Table 4 presents the susceptibility profiles of *C. albicans* isolates against anidulafungin, caspofungin, fluconazole, micafungin, and voriconazole.

Table 4: Susceptibility profiles of *C. albicans* isolates against anidulafungin, caspofungin, fluconazole, micafungin and voriconazole (n = 72).

Antifungal	Susceptibility profile		
	Susceptible	**SDD**	**Resistant**
Anidulafungin	66 (91.7%)	1 (1.4%)	5 (6.9%)
Caspofungin	68 (94.4%)	2 (2.8%)	2 (2.8%)
Fluconazole	62 (86.1%)	0 (0%)	10 (13.9%)
Micafungin	65 (90.3%)	4 (5.5%)	3 (4.2%)
Voriconazole	62 (86%)	5 (7%)	5 (7%)

SDD - susceptible-dose-dependent

Correlation between susceptibility profiles and genotypes

Table 5 presents the correlation between anidulafungin susceptibility patterns and *C. albicans* genotypes. Among the 66 isolates that were susceptible to anidulafungin, 41/66 (62.1%) were classified as genotype A, 18/66 (27.3%) as genotype B, and 7/66 (10.6%) as genotype C. The single isolate that was susceptible-dose-dependent to anidulafungin (1/1; 100%) was assigned genotype B. Among the five isolates resistant to anidulafungin, 4/5 (80%) were classified as genotype A and 1/5 (20%) as genotype C. Table 6 illustrates the correlation between caspofungin susceptibility patterns and *C. albicans* genotypes. Among the 68 isolates that were susceptible to caspofungin, 43/68 (63.2%) were classified as genotype A, 19/68 (28%) as genotype B, and 6/68 (8.8%) as genotype C. The isolates that were susceptible-dose-dependent to caspofungin (2/2; 100%) were both assigned to genotype A. Additionally, the isolates resistant to caspofungin (2/2; 100%) were both classified as genotype C.

Table 7 presents the correlation between fluconazole susceptibility patterns and *C. albicans* genotypes. Among the 62 isolates that were susceptible to fluconazole, 37/62 (59.7%) were classified as genotype A, 19/62 (30.6%) as genotype B, and 6/62 (9.7%) as genotype C. Of the 10 isolates that were resistant to fluconazole, 8/10 (80%) were assigned to genotype A, while 2/10 (20%) were assigned to genotype C. Table 8 illustrates the correlation between micafungin susceptibility patterns and *C. albicans* genotypes. Among the 65 isolates that were susceptible to micafungin, 40/65 (61.5%) were classified as genotype A, 18/65 (27.7%) as genotype B, and 7/65 (10.8%) as genotype C. Of the four isolates that were susceptible-dose-dependent to micafungin, 3/4 (75%) were assigned to genotype A, while 1/4 (25%) was assigned to genotype B. Among the three isolates that were resistant to micafungin, 2/3 (66.7%) were classified as genotype A and 1/3 (33.3%) as genotype C.

Table 9 presents the correlation between voriconazole susceptibility patterns and *C. albicans* genotypes. Among the 62 isolates that were susceptible to voriconazole, 37/62 (59.7%) were classified as genotype A, 19/62 (30.6%) as genotype B, and 6/62 (9.7%) as genotype C. All isolates that were susceptible-

dose-dependent to voriconazole (5/5; 100%) were assigned to genotype A. Among the five isolates that were resistant to voriconazole, 3/5 (60%) were classified as genotype A, while 2/5 (40%) were classified as genotype C.

Table 5: Correlation between the susceptibility profile of anidulafungin and *C. albicans* genotypes.

Genotype	Susceptibility pattern		
	Susceptible (*n* = 66)	**SDD (*n* = 1)**	**Resistant (*n* = 5)**
A	41 (62.1%)	0 (0%)	4 (80%)
B	18 (27.3%)	1 (100%)	0 (0%)
C	7 (10.6%)	0 (0%)	1 (20%)

SDD - susceptible-dose-dependent

Table 6: Correlation between the susceptibility profile of caspofungin and *C. albicans* genotypes.

Genotype	Susceptibility pattern		
	Susceptible (*n* = 68)	**SDD (*n* = 2)**	**Resistant (*n* = 2)**
A	43 (63.2%)	2 (100%)	0 (0%)
B	19 (28%)	0 (0%)	0 (0%)
C	6 (8.8%)	0 (0%)	2 (100%)

SDD - susceptible-dose-dependent

Table 7: Correlation between the susceptibility profile of fluconazole and *C. albicans* genotypes.

Genotype	Susceptibility pattern	
	Susceptible (*n* = 62)	**Resistant (*n* = 10)**
A	37 (59.7%)	8 (80%)
B	19 (30.6%)	0 (0%)
C	6 (9.7%)	2 (20%)

SDD - susceptible-dose-dependent

Table 8: Correlation between the susceptibility profile of micafungin and *C. albicans* genotypes.

Genotype	Susceptibility pattern		
	Susceptible (*n* = 65)	**SDD (*n* = 4)**	**Resistant (*n* = 3)**
A	40 (61.5%)	3 (75%)	2 (66.7%)
B	18 (27.7%)	1 (25%)	0 (0%)
C	7 (10.8%)	0 (0%)	1 (33.3%)

SDD - susceptible-dose-dependent

Table 9: Correlation between the susceptibility profile of voriconazole and *C. albicans* genotypes.

Genotype	Susceptibility pattern		
	Susceptible (*n* = 62)	**SDD (*n* = 5)**	**Resistant (*n* = 5)**
A	37 (59.7%)	5 (100%)	3 (60%)
B	19 (30.6%)	0 (0%)	0 (0%)
C	6 (9.7%)	0 (0%)	2 (40%)

SDD - susceptible-dose-dependent

Discussion

Candida species account for a substantial proportion of fungal infections related to women's healthcare globally.[2] *C. albicans* is responsible for over 80% of yeast infections.[15] This study aimed to link the distribution of *C. albicans* genotypes with the antifungal susceptibility profiles of anidulafungin, caspofungin, fluconazole, micafungin and voriconazole in 72 *C. albicans* isolates obtained from vaginal swabs of pregnant and non-pregnant patients at the Victoria Mxenge Hospital in Durban, South Africa.

ABC genotyping analysis of *25S rDNA* is a molecular typing technique widely used to detect variations among *Candida* species, playing an important role in linking specific genotypes to antifungal resistance and serving as a valuable tool for epidemiological research.[7,16] In the current study, the majority of the 72 isolates were identified as genotype A (62.5%), with genotype B (26.4%) and genotype C (11.1%) following. These findings align with those of another study, which reported genotype A as the most prevalent among all *C. albicans* isolates (54.69%), followed by genotype B (34.38%) and genotype C (10.94%).[17] This study is consistent with findings from research conducted in Iraq on patients with VVC. Among the 54 *C. albicans* isolates examined in that study, genotype A was the most prevalent, accounting for 50%, followed by genotype B at 29.62%, and genotype C at 20.37%.[18] A study conducted in northeast Brazil on women with vaginal *Candida* infections also identified genotype A as the most prevalent following genotype C, at prevalence rates of 93.6% and 6.4%, respectively. Notably, genotypes B and D were not detected in that study.[19] However, in a study conducted in Palestine, 55%

of the vaginal *Candida* isolates had genotype C, followed by genotypes A and B with prevalence rates at 32.4% and 12.6%, respectively.[20] An Iranian study also found that the most common genotype was genotype C (83.5%), followed by genotype B (12.6%) and genotype A (3.9%).[21] The variations in genotypes observed may be attributed to differences in geographical regions.

The resistance of *Candida* species to antifungal treatments remains a major challenge in managing fungal infections.[22] In this study, we assessed the *in vitro* susceptibility of 72 *C. albicans* isolates to five antifungal agents: anidulafungin, caspofungin, fluconazole, micafungin, and voriconazole. Anidulafungin, caspofungin, and micafungin are part of the Echinocandin class of antifungals, while fluconazole and voriconazole are classified as azole antifungals. Resistance to these drugs can lead to treatment failures. *Candida* species show the highest prevalence of resistance to azole antifungals. Vaginal *C. albicans* isolates are known to exhibit high resistance rates to fluconazole.[23] Among these isolates, 91.7% demonstrated susceptibility to anidulafungin, while 6.9% exhibited resistance. For caspofungin, 94.4% were susceptible, with 2.8% showing resistance. Regarding fluconazole, 86.1% of isolates were susceptible, and 13.9% were resistant. Micafungin showed a susceptibility rate of 90.3%, with 4.2% resistance. Lastly, voriconazole had a susceptibility rate of 86%, and 7% of isolates were resistant. Caspofungin and micafungin demonstrated lower resistance rates compared to anidulafungin, fluconazole, and voriconazole. Among the antifungal agents tested, fluconazole exhibited the highest resistance rates.

These results align with findings from another study, which reported resistance rates for caspofungin, fluconazole, and voriconazole at 1%, 9%, and 6%, respectively.[24] Similarly, a study conducted in China demonstrated low resistance rates against anidulafungin, caspofungin, and micafungin, while higher resistance rates were noted for fluconazole and voriconazole.[25] High resistance rates to fluconazole and voriconazole have also been observed in additional studies from China and Bulgaria.[26,27] In contrast, research conducted in Tanzania revealed lower resistance rates, with 3.1% for fluconazole and 3.6% for voriconazole.[28] Research on antifungal resistance among *Candida* species is valuable as it offers up-to-date data on resistance patterns, aiding in the evaluation of empirical treatment guidelines. Variations in antifungal susceptibility profiles among *C. albicans* can be attributed to geographic differences and variations in the populations studied.

The current study also described the correlation between the *C. albicans* genotypes detected in relation to the susceptibility profiles of anidulafungin, caspofungin, fluconazole, micafungin, and voriconazole. Of the 66 isolates that were susceptible to anidulafungin, 62.1% belonged to genotype A, 27.3% were genotype B, and 10.6% were genotype C. Among the five isolates resistant to anidulafungin, 80% were categorized as genotype A, with the remaining 20% classified as genotype C. For caspofungin susceptibility, 63.2% of the 68 isolates were identified as genotype A, 28% as genotype B, and 8.8% as genotype C, while both caspofungin-resistant isolates were genotype C. Of the 62 fluconazole-susceptible isolates, 59.7% were genotype A, 30.6% were genotype B, and 9.7% were genotype C; for

the 10 fluconazole-resistant isolates, 80% were identified as genotype A, and 20% as genotype C. Regarding micafungin, 61.5% of the 65 susceptible isolates were genotype A, 27.7% were genotype B, and 10.8% were genotype C, whereas among the three micafungin-resistant isolates, 66.7% were genotype A and 33.3% were genotype C. Finally, of the 62 isolates susceptible to voriconazole, 59.7% were genotype A, 30.6% were genotype B, and 9.7% were genotype C; among the five voriconazole-resistant isolates, 60% were genotype A and 40% were genotype C.

The study demonstrated that most isolates susceptible to the five antifungal agents were predominantly of genotype A, followed by genotype B, and then genotype C. Among the isolates resistant to anidulafungin, fluconazole, micafungin, and voriconazole, most were identified as genotype A, with the remainder being genotype C. Notably, all isolates resistant to caspofungin were of genotype C. No resistant isolates were classified as genotype B, demonstrating that this genotype had a 0% resistance rate, the lowest among the genotypes studied. However, other studies conducted in China assessing *C. albicans* genotypes against azole antifungals revealed that genotype A isolates showed lower resistance rates than those of genotype B.[23,29] Another study conducted in Jordan found that all *Candida* isolates collected from women with vaginal candidiasis were susceptible to fluconazole, resulting in a 0% resistance rate among genotypes A, B, and C.[30] Lastly, a study conducted in China revealed that *C. albicans 25S rDNA* genotypes belonging to group A exhibited significantly lower susceptibility rates to fluconazole compared to genotypes B and C.[31]

To our knowledge, no South African studies have linked the genotypes of *C. albicans* isolates from pregnant and non-pregnant women to the antifungal susceptibility profiles of anidulafungin, caspofungin, fluconazole, micafungin, and voriconazole. In this study, the majority of *C. albicans* isolates were identified as genotype A and exhibited resistance to anidulafungin, fluconazole, micafungin, and voriconazole, while caspofungin-resistant isolates were all classified as genotype C. This indicates that genotypes A and C are more virulent than genotype B. Currently, there is limited research on the correlation between *Candida* genotypes and antifungal susceptibility patterns, a gap in the literature that this study addresses.

Study limitations

The study faced a few limitations: the small sample size may have been a reason for the study being unable to detect non-*albicans Candida* species. Additionally, since the research was carried out in a single geographical area and the participants were all recruited from one clinic, it may not fully represent the broader population. However, given that Victoria Mxenge Hospital serves as a central tertiary hospital, it does reflect a wider portion of Durban's population.

Conclusion

In conclusion, the majority of *C. albicans* isolates collected from both pregnant and non-pregnant South African women demonstrated susceptibility to anidulafungin, caspofungin, fluconazole, micafungin, and voriconazole. Genotype A was the most prevalent *C. albicans* genotype among women in Durban, South Africa. The isolates susceptible to the five antifungal agents were mainly genotype A, followed by genotype B, and then genotype C. Among isolates showing resistance to anidulafungin, fluconazole, micafungin, and voriconazole, the majority were classified as genotype A, with the remaining resistant isolates identified as genotype C. All isolates resistant to caspofungin belonged to genotype C. No resistance to any tested antifungal drugs was found among isolates of genotype B, suggesting it is the least virulent strain of *C. albicans*. Additional research is necessary to investigate the occurrence of other genotypes, such as genotype D, and to establish their correlation with antifungal resistance patterns. Future studies can now focus on the mechanisms behind resistance in local isolates. Currently, antifungal resistance patterns for commonly used treatments of *Candida* infections are not being monitored in our local setting, highlighting a need for resistance surveillance to mitigate the risk of future untreatable infections.

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Conflict of interest

The authors declare no conflict of interest.

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Ethical approval

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (reference number BREC/00005995/2023).

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References

1. Rudramurthy SM and Singh S. Candida infections in immunocompetent hosts: pathogenesis and diagnosis. *Curr. Fungal Infect. Rep.* 2020; 14:233-45.

2. Sobel JD. Recurrent vulvovaginal candidiasis. *Am. J. Obstet. Gynecol.* 2016; 214(1):15-21.

3. de Cássia Orlandi Sardi J, Silva DR, Anibal PC, et al. Vulvovaginal candidiasis: epidemiology and risk factors, pathogenesis, resistance, and new therapeutic options. *Curr. Fungal Infect. Rep.* 2021; 15:32-40.

4. Omrani S, Pecen L, Rajek P, et al. Prevalence of invasive and superficial Candida infections in Africa and the Middle East; a systematic review and meta-analysis. *Am. Soc. Microbiol.* 2014; 17-20. doi: 10.1016/j.mycmed.2019.07.006.

5. Bongomin F, Gago S, Oladele RO, et al. Global and multi-national prevalence of fungal diseases—estimate precision. *J. Fungus.* 2017; 3:57. doi: 10.3390/jof3040057

6. Sim M, Logan S and Goh LH. Vaginal discharge: evaluation and management in primary care. *Singap. Med. J.* 2020; 61(6):297.

7. Fornari G, Vicente VA, Gomes RR, et al. Susceptibility and molecular characterization of Candida species from patients with vulvovaginitis. *Braz. J. Microbiol.* 2016; 47(2):373-380.

8. Lyon JP, Moraes K, Moreira LM, et al. Candida albicans: genotyping methods and clade related phenotypic characteristics. *Braz. J. Microbiol.* 2010; 41:841-9.

9. Jafarian H, Gharaghani M, Seyedian SS, et al. Genotyping, antifungal susceptibility, enzymatic activity, and phenotypic variation in Candida albicans from esophageal candidiasis. *J. Clin. Lab. Analy.* 2021; 35(7):e23826.

10. Lee Y, Puumala E, Robbins N, et al. Antifungal drug resistance: molecular mechanisms in Candida albicans and beyond. *Chem. Rev.* 2020; 121(6):3390-411.

11. Wiederhold NP. The antifungal arsenal: alternative drugs and future targets. *Int. J. Antimicrob. Agents.* 2018; 51(3):333-9.

12. Bhattacharya S, Sae-Tia S, and Fries BC. Candidiasis and mechanisms of antifungal resistance. *J. Antibiot.* 2020; 9:312. doi:10.3390/antibiotics9060312

13. Terrence DC and Ihrke DM. Further studies of the germ-tube test for Candida albicans identification. *Am. J. Clin. Pathol.* 1971; 55:733-734. doi: 10.1093/ajcp/55.6.733.

14. Mashaly GES and Zeid MS. Candida albicans genotyping and relationship of virulence factors with fluconazole tolerance in infected pediatric patients. *Infect. Drug. Resist*. 2022; 15:2035-2043. doi: 10.2147/IDR.S344998

15. Talapko J, Juzbašić M, Matijević T, et al. Candida albicans—the virulence factors and clinical manifestations of infection. *J. Fungi.* 2021; 7(2):79.

16. Tapia CV, Hermosilla G, Fortes P, et al. Genotyping and persistence of Candida albicans from pregnant women with vulvovaginal candidiasis. *Mycopathologia.* 2017; 182:339-47.

17. Shekhany KAM. Isolation and genotyping of Candida albicans involved in vaginal candidiasis among pregnant women in Sulaymaniyah and Erbil cities. *Zanco. J. Med. Sci.* 2021; 25(1):493-502. doi: 10.15218/zjms.2021.012

18. Mohammed AN, Bander K and Hamada T. Genotype Comparisons of Candida albicans from patients with vulvovaginal candidiasis. *Egypt. Aca. J. Biol. Sci.* 2016; 8(1):1-5. doi: 10.21608/eajbsg.2016.16472

19. De Medeiros APM, De Melo VAP, Goncalves SS, et al. Genetic relationships among vaginal and anal isolates of Candida albicans from women with vulvovaginal candidiasis in north-east Brazil. *J. Med. Microbiol.* 2014; 63(11):1436-1445. doi:10.1099/jmm.0.076604-0

20. Mohammed SAS, Rana MJ, Nihad HAA, et al. Genotyping and antifungal susceptibility of Candida albicans strains from patients with vulvovaginal and cutaneous candidiasis in Palestine. *Afr. J. Microbiol. Res*. 2015; 9(13):952-959.

21. Gharaghani M, Shabanzadeh M, Jafarian H, et al. ABC typing and extracellular enzyme production of Candida albicans isolated from Candida vulvovaginitis. *J. Clin. Lab. Analy.* 2022; 36(1):e24117. doi: 10.1002/jcla.24117

22. Richter SS, Galask RP, Messer SA, et al. Antifungal susceptibilities of Candida species causing vulvovaginitis and epidemiology of recurrent cases. *J. Clin. Microbiol.* 2005; 43:2155-2162.

23. Liu XP, Fan SR, Bai FY, et al. Antifungal susceptibility and genotypes of Candida albicans strains from patients with vulvovaginal candidiasis. *Mycoses.* 2009; 52:24-28.

24. Al-Ameri AD, Zghair AS, Al-Nuaimi NB, et al. Evaluation of Susceptibility of Candida species to Six Antifungal Drugs in Iraqi Specimens. *J. Commun. Dis.* 2024; 56(2):53-61.

25. Yan L, Wang XD, Seyedmousavi S, et al. Antifungal Susceptibility Profile of Candida albicans isolated from vulvovaginal candidiasis in Xinjiang province of China. *Mycopathologia.* 2019; 184:413-422. doi: 10.1007/s11046-018-0305-2

26. Shi Y, Zhu Y, Fan S, et al. Molecular identification and antifungal susceptibility profile of yeast from vulvovaginal candidiasis. *BMC. Infect. Dis.* 2020; 20:287. doi: 10.1186/s12879-020-04985-w

27. Hitkova HY, Georgieva DS, Hristova PM, et al. Antifungal Susceptibility of Candida albicans isolates at a tertiary care hospital in Bulgaria. *Jundishapur. J. Microbiol.* 2019; 12(7):e92079. doi: 10.5812/jjm.92079

28. Mushi MF, Bader O, Bii C, et al. Virulence and susceptibility patterns of clinical Candida spp. isolates from a tertiary hospital, Tanzania. *Med. Mycol.* 2019; 57(5):566-572. doi: 10.1093/mmy/myy107

29. Ge SH, Wan Z, Li J, et al. Correlation between azole susceptibilities, genotypes, and ERG11 mutations in Candida albicans isolates associated with vulvovaginal candidiasis in China. *Antimicro. Ag. Chemo*. 2010; 54(8):3126-3131. doi:10.1128/aac.00118-10

30. Al-Groom RM, Ali RRM and Abu Shaqra QM. Genotypes analysis and antifungal susceptibility of Candida albicans strains isolated from women with vaginal candidiasis in Jordan using PCR targeting 25SrDNA and ALT repeat sequences of the RPS. *Pak. J. Med. Sci.* 2024; 40(8):1619-1624. doi: 10.12669/pjms.40.8.9811

31. Wang M, Cao Y, Xia M, et al. Virulence and antifungal susceptibility of microsatellite genotypes of Candida albicans from superficial and deep locations. *Yeast.* 2019. doi: 10.1002/yea.3397

Supplementary Material

Supplementary Table 1: *C. albicans* isolates: MICs and susceptibility profiles to anidulafungin.

Isolate name	MIC (µg/ml)	Susceptibility profile
ATCC	0.12	Susceptible
ZMO1	0.06	Susceptible
ZMO10	0.12	Susceptible
ZMO11	0.12	Susceptible
ZMO12	< 0.015	Susceptible
ZMO14	0.12	Susceptible
ZMO17	0.12	Susceptible

ZMO18	0.06	Susceptible
ZMO20	0.12	Susceptible
ZMO21	0.03	Susceptible
ZMO23	0.03	Susceptible
ZMO25	0.12	Susceptible
ZMO27	0.25	Susceptible
ZMO28	0.12	Susceptible
ZMO29	0.12	Susceptible
ZMO30	0.12	Susceptible
ZMO32	< 0.015	Susceptible
ZMO34	< 0.015	Susceptible
ZMO35	0.12	Susceptible
ZMO37	0.06	Susceptible
ZMO40	0.06	Susceptible
ZMO41	0.03	Susceptible
ZMO42	< 0.015	Susceptible
ZMO43	0.12	Susceptible
ZMO44	0.12	Susceptible
ZMO47	0.12	Susceptible
ZMO53	< 0.015	Susceptible
ZMO54	0.12	Susceptible
ZMO56	0.12	Susceptible
ZMO58	0.12	Susceptible
ZMO59	0.03	Susceptible
ZMO60	0.03	Susceptible
ZMO62	012	Susceptible
ZMO63	0.03	Susceptible
ZMO65	0.12	Susceptible
ZMO67	0.06	Susceptible
ZMO68	1	Resistant
ZMO69	< 0.015	Susceptible
ZMO71	< 0.015	Susceptible
ZMO72	0.12	Susceptible
ZMO75	0.06	Susceptible
ZMO77	0.03	Susceptible
ZMO79	0.12	Susceptible

ZMO80	0.12	Susceptible
ZMO81	< 0.015	Susceptible
ZMO82	< 0.015	Susceptible
ZMO83	0.12	Susceptible
ZMO84	0.12	Susceptible
ZMO85	2	Resistant
ZMO86	< 0.015	Susceptible
ZMO87	0.5	Susceptible-dose-dependent
ZMO88	0.12	Susceptible
ZMO89	0.12	Susceptible
ZMO91	0.06	Susceptible
ZMO94	0.06	Susceptible
ZMO95	0.12	Susceptible
ZMO96	0.12	Susceptible
ZMO97	0.06	Susceptible
ZMO98	0.06	Susceptible
ZMO99	< 0.015	Susceptible
ZMO102	< 0.015	Susceptible
ZMO103	0.12	Susceptible
ZMO107	< 0.015	Susceptible
ZMO110	< 0.015	Susceptible
ZMO119	< 0.015	Susceptible
ZMO128	0.12	Susceptible
ZMO132	< 0.015	Susceptible
ZMO135	4	Resistant
ZMO141	< 0.015	Susceptible
ZMO142	1	Resistant
ZMO145	1	Resistant
ZMO146	0.06	Susceptible
ZMO147	0.06	Susceptible

MICs - minimum inhibitory concentrations

Supplementary Table 2: *C. albicans* isolates: MICs and susceptibility profiles to caspofungin.

Isolate name	MIC (µg/ml)	Susceptibility profile
ATCC	0.12	Susceptible
ZMO1	0.06	Susceptible
ZMO10	0.5	Susceptible-dose-dependent
ZMO11	0.25	Susceptible
ZMO12	0.06	Susceptible
ZMO14	0.06	Susceptible
ZMO17	0.06	Susceptible
ZMO18	0.03	Susceptible
ZMO20	0.12	Susceptible
ZMO21	0.12	Susceptible
ZMO23	0.06	Susceptible
ZMO25	0.06	Susceptible
ZMO27	0.12	Susceptible
ZMO28	0.06	Susceptible
ZMO29	0.12	Susceptible
ZMO30	0.12	Susceptible
ZMO32	< 0.008	Susceptible
ZMO34	0.12	Susceptible
ZMO35	0.12	Susceptible
ZMO37	0.03	Susceptible
ZMO40	0.06	Susceptible
ZMO41	0.06	Susceptible
ZMO42	1	Resistant
ZMO43	0.12	Susceptible
ZMO44	0.12	Susceptible
ZMO47	0.12	Susceptible
ZMO53	0.03	Susceptible
ZMO54	0.12	Susceptible
ZMO56	0.06	Susceptible
ZMO58	0.12	Susceptible
ZMO59	0.06	Susceptible
ZMO60	0.06	Susceptible
ZMO62	0.06	Susceptible
ZMO63	0.03	Susceptible

ZMO65	0.06	Susceptible
ZMO67	0.06	Susceptible
ZMO68	0.5	Susceptible-dose-dependent
ZMO69	< 0.008	Susceptible
ZMO71	< 0.008	Susceptible
ZMO72	0.06	Susceptible
ZMO75	0.03	Susceptible
ZMO77	0.12	Susceptible
ZMO79	0.12	Susceptible
ZMO80	0.12	Susceptible
ZMO81	0.06	Susceptible
ZMO82	0.06	Susceptible
ZMO83	0.12	Susceptible
ZMO84	0.06	Susceptible
ZMO85	< 0.008	Susceptible
ZMO86	< 0.008	Susceptible
ZMO87	0.25	Susceptible
ZMO88	0.06	Susceptible
ZMO89	0.12	Susceptible
ZMO91	0.06	Susceptible
ZMO94	0.06	Susceptible
ZMO95	0.12	Susceptible
ZMO96	0.03	Susceptible
ZMO97	0.03	Susceptible
ZMO98	0.06	Susceptible
ZMO99	0.06	Susceptible
ZMO102	0.03	Susceptible
ZMO103	0.06	Susceptible
ZMO107	< 0.008	Susceptible
ZMO110	0.03	Susceptible
ZMO119	< 0.008	Susceptible
ZMO128	0.12	Susceptible
ZMO132	< 0.008	Susceptible
ZMO135	8	Resistant
ZMO141	< 0.008	Susceptible
ZMO142	0.25	Susceptible

ZMO145	0.25	Susceptible
ZMO146	0.12	Susceptible
ZMO147	0.06	Susceptible

MICs - minimum inhibitory concentrations

Supplementary Table 3: *C. albicans* isolates: MICs and susceptibility profiles to fluconazole.

Isolate name	MIC (µg/ml)	Susceptibility profile
ATCC	2	Susceptible
ZMO1	0.25	Susceptible
ZMO10	64	Resistant
ZMO11	32	Resistant
ZMO12	0.25	Susceptible
ZMO14	0.5	Susceptible
ZMO17	0.5	Susceptible
ZMO18	0.25	Susceptible
ZMO20	0.5	Susceptible
ZMO21	< 0.12	Susceptible
ZMO23	0.25	Susceptible
ZMO25	0.5	Susceptible
ZMO27	0.25	Susceptible
ZMO28	0.25	Susceptible
ZMO29	0.5	Susceptible
ZMO30	0.12	Susceptible
ZMO32	< 0.12	Susceptible
ZMO34	0.12	Susceptible
ZMO35	64	Resistant
ZMO37	0.5	Susceptible
ZMO40	0.25	Susceptible
ZMO41	0.5	Susceptible
ZMO42	< 0.12	Susceptible
ZMO43	0.5	Susceptible
ZMO44	32	Resistant
ZMO47	4	Susceptible
ZMO53	< 0.12	Susceptible
ZMO54	0.5	Susceptible

ZMO56	0.25	Susceptible
ZMO58	0.25	Susceptible
ZMO59	0.5	Susceptible
ZMO60	< 0.12	Susceptible
ZMO62	2	Susceptible
ZMO63	0.25	Susceptible
ZMO65	8	Resistant
ZMO67	0.5	Susceptible
ZMO68	0.5	Susceptible
ZMO69	< 0.12	Susceptible
ZMO71	< 0.12	Susceptible
ZMO72	0.25	Susceptible
ZMO75	0.5	Susceptible
ZMO77	< 0.12	Susceptible
ZMO79	2	Susceptible
ZMO80	0.5	Susceptible
ZMO81	0.5	Susceptible
ZMO82	< 0.12	Susceptible
ZMO83	0.25	Susceptible
ZMO84	0.5	Susceptible
ZMO85	8	Resistant
ZMO86	< 0.12	Susceptible
ZMO87	2	Susceptible
ZMO88	1	Susceptible
ZMO89	0.5	Susceptible
ZMO91	0.25	Susceptible
ZMO94	0.25	Susceptible
ZMO95	0.5	Susceptible
ZMO96	< 0.12	Susceptible
ZMO97	0.5	Susceptible
ZMO98	0.5	Susceptible
ZMO99	< 0.12	Susceptible
ZMO102	< 0.12	Susceptible
ZMO103	2	Susceptible
ZMO107	< 0.12	Susceptible
ZMO110	< 0.12	Susceptible

ZMO119	< 0.12	Susceptible
ZMO128	32	Resistant
ZMO132	< 0.12	Susceptible
ZMO135	> 256	Resistant
ZMO141	< 0.12	Susceptible
ZMO142	8	Resistant
ZMO145	16	Resistant
ZMO146	< 0.12	Susceptible
ZMO147	0.25	Susceptible

MICs - minimum inhibitory concentrations

Supplementary Table 4: *C. albicans* isolates: MICs and susceptibility profiles to micafungin.

Isolate name	MIC (µg/ml)	Susceptibility profile
ATCC	0.015	Susceptible
ZMO1	< 0.008	Susceptible
ZMO10	0.25	Susceptible
ZMO11	0.015	Susceptible
ZMO12	0.015	Susceptible
ZMO14	0.015	Susceptible
ZMO17	0.015	Susceptible
ZMO18	0.015	Susceptible
ZMO20	0.015	Susceptible
ZMO21	0.015	Susceptible
ZMO23	0.015	Susceptible
ZMO25	0.015	Susceptible
ZMO27	0.015	Susceptible
ZMO28	< 0.008	Susceptible
ZMO29	0.5	Susceptible-dose-dependent
ZMO30	0.015	Susceptible
ZMO32	< 0.008	Susceptible
ZMO34	0.015	Susceptible
ZMO35	0.015	Susceptible
ZMO37	< 0.008	Susceptible
ZMO40	< 0.008	Susceptible
ZMO41	< 0.008	Susceptible

ZMO42	< 0.008	Susceptible
ZMO43	0.015	Susceptible
ZMO44	0.015	Susceptible
ZMO47	0.015	Susceptible
ZMO53	< 0.008	Susceptible
ZMO54	0.015	Susceptible
ZMO56	0.015	Susceptible
ZMO58	< 0.008	Susceptible
ZMO59	0.015	Susceptible
ZMO60	< 0.008	Susceptible
ZMO62	0.015	Susceptible
ZMO63	< 0.008	Susceptible
ZMO65	0.06	Susceptible
ZMO67	< 0.008	Susceptible
ZMO68	1	Resistant
ZMO69	< 0.008	Susceptible
ZMO71	< 0.008	Susceptible
ZMO72	0.015	Susceptible
ZMO75	< 0.008	Susceptible
ZMO77	0.03	Susceptible
ZMO79	0.06	Susceptible
ZMO80	0.03	Susceptible
ZMO81	< 0.008	Susceptible
ZMO82	< 0.008	Susceptible
ZMO83	0.015	Susceptible
ZMO84	0.03	Susceptible
ZMO85	2	Resistant
ZMO86	< 0.008	Susceptible
ZMO87	0.5	Susceptible-dose-dependent
ZMO88	0.015	Susceptible
ZMO89	0.015	Susceptible
ZMO91	< 0.008	Susceptible
ZMO94	< 0.008	Susceptible
ZMO95	0.03	Susceptible
ZMO96	0.015	Susceptible
ZMO97	0.015	Susceptible

ZMO98	0.015	Susceptible
ZMO99	< 0.008	Susceptible
ZMO102	< 0.008	Susceptible
ZMO103	0.03	Susceptible
ZMO107	< 0.008	Susceptible
ZMO110	< 0.008	Susceptible
ZMO119	< 0.008	Susceptible
ZMO128	0.015	Susceptible
ZMO132	< 0.008	Susceptible
ZMO135	> 8	Resistant
ZMO141	< 0.008	Susceptible
ZMO142	0.5	Susceptible-dose-dependent
ZMO145	0.5	Susceptible-dose-dependent
ZMO146	0.015	Susceptible
ZMO147	< 0.008	Susceptible

MICs - minimum inhibitory concentrations

Supplementary Table 5: *C. albicans* isolates: MICs and susceptibility profiles to voriconazole.

Isolate name	MIC (µg/ml)	Susceptibility profile
ATCC	0.06	Susceptible
ZMO1	< 0.008	Susceptible
ZMO10	1	Resistant
ZMO11	0.5	Susceptible-dose-dependent
ZMO12	< 0.008	Susceptible
ZMO14	0.015	Susceptible
ZMO17	< 0.008	Susceptible
ZMO18	< 0.008	Susceptible
ZMO20	0.015	Susceptible
ZMO21	< 0.008	Susceptible
ZMO23	< 0.008	Susceptible
ZMO25	< 0.008	Susceptible
ZMO27	< 0.008	Susceptible
ZMO28	< 0.008	Susceptible
ZMO29	< 0.008	Susceptible
ZMO30	< 0.008	Susceptible

ZMO32	< 0.008	Susceptible
ZMO34	< 0.008	Susceptible
ZMO35	2	Resistant
ZMO37	0.015	Susceptible
ZMO40	< 0.008	Susceptible
ZMO41	< 0.008	Susceptible
ZMO42	< 0.008	Susceptible
ZMO43	< 0.008	Susceptible
ZMO44	1	Resistant
ZMO47	0.25	Susceptible-dose-dependent
ZMO53	< 0.008	Susceptible
ZMO54	< 0.008	Susceptible
ZMO56	< 0.008	Susceptible
ZMO58	< 0.008	Susceptible
ZMO59	< 0.008	Susceptible
ZMO60	< 0.008	Susceptible
ZMO62	0.12	Susceptible
ZMO63	< 0.008	Susceptible
ZMO65	0.12	Susceptible
ZMO67	< 0.008	Susceptible
ZMO68	< 0.008	Susceptible
ZMO69	< 0.008	Susceptible
ZMO71	< 0.008	Susceptible
ZMO72	< 0.008	Susceptible
ZMO75	< 0.008	Susceptible
ZMO77	< 0.008	Susceptible
ZMO79	0.25	Susceptible-dose-dependent
ZMO80	< 0.008	Susceptible
ZMO81	< 0.008	Susceptible
ZMO82	< 0.008	Susceptible
ZMO83	< 0.008	Susceptible
ZMO84	0.015	Susceptible
ZMO85	0.12	Susceptible
ZMO86	< 0.008	Susceptible
ZMO87	0.03	Susceptible
ZMO88	0.06	Susceptible

ZMO89	< 0.008	Susceptible
ZMO91	< 0.008	Susceptible
ZMO94	< 0.008	Susceptible
ZMO95	< 0.008	Susceptible
ZMO96	< 0.008	Susceptible
ZMO97	< 0.008	Susceptible
ZMO98	< 0.008	Susceptible
ZMO99	< 0.008	Susceptible
ZMO102	< 0.008	Susceptible
ZMO103	0.12	Susceptible
ZMO107	< 0.008	Susceptible
ZMO110	< 0.008	Susceptible
ZMO119	< 0.008	Susceptible
ZMO128	1	Resistant
ZMO132	< 0.008	Susceptible
ZMO135	> 8	Resistant
ZMO141	< 0.008	Susceptible
ZMO142	0.5	Susceptible-dose-dependent
ZMO145	0.5	Susceptible-dose-dependent
ZMO146	< 0.008	Susceptible
ZMO147	< 0.008	Susceptible

MICs - minimum inhibitory concentrations

BRIDGE

Chapter 2 demonstrated that *C. albicans* isolates circulating in this population show distinct genotypic profiles and varying susceptibility to fluconazole, with evidence suggesting possible associations between genotype and resistance. While these findings offer important epidemiological and phenotypic insights, the underlying molecular mechanisms responsible for fluconazole resistance remain unclear. Genotypic classification alone cannot fully explain the observed resistance patterns, underscoring the need for a more comprehensive analysis at the gene expression level. Chapter 3, therefore, expands the investigation by examining *ERG11* gene expression and phylogenetic relationships among resistant and susceptible isolates. By quantifying transcriptional changes and exploring evolutionary patterns, this chapter provides critical molecular evidence that complements the genotyping data. Together, these analyses clarify the biological basis of azole resistance within this cohort and contribute to a more comprehensive understanding of strain-specific resistance mechanisms.

CHAPTER 3

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***ERG11* overexpression and genetic divergence drive fluconazole resistance in clinical *Candida albicans*: A droplet digital polymerase chain reaction and phylogenetic analysis**

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Abstract

Fluconazole resistance in *Candida albicans* (*C. albicans*) presents a growing clinical challenge, particularly in regions where azoles remain the primary therapeutic option. This study investigated the molecular basis of fluconazole resistance among *C. albicans* clinical isolates by integrating antifungal susceptibility testing, quantitative gene-expression profiling, and phylogenetic analysis of the lanosterol 14-α-demethylase (*ERG11*) gene. Eleven isolates (ten fluconazole-resistant and one fluconazole-susceptible) were analysed using Sensititre™ YeastOne™ assays, droplet digital polymerase chain reaction (ddPCR), and sequence-based bioinformatic tools. As expected, based on previous characterisation, susceptibility testing confirmed high-level resistance across most isolates, with minimum inhibitory concentrations (MICs) ranging from 8 µg/ml to >256 µg/ml. ddPCR quantification revealed *ERG11* overexpression in nine resistant isolates, with fold-change values ranging from 1.31 to 3.73 compared to the susceptible control (ZMO28). Isolates ZMO10 and ZMO135 exhibited an upregulation of *ERG11* (>3-fold), correlating with the highest resistance levels (MICs ≥64 µg/ml). Conversely, isolate ZMO128 demonstrated markedly reduced *ERG11* expression (0.15-fold) despite phenotypic resistance with an elevated MIC of 32 µg/ml, suggesting alternative mechanisms such as efflux pump activation or biofilm-associated tolerance. Phylogenetic reconstruction of *ERG11* sequences revealed a major resistant clade with two internal sub-clusters, reflecting shared evolutionary pathways among highly expressed isolates, while ZMO128 formed a genetically distinct branch. These findings suggest that fluconazole resistance is primarily driven by *ERG11* upregulation, although additional pathways contribute to resistance heterogeneity. This study underscores the importance of integrating molecular diagnostics into antifungal stewardship, particularly in settings where azole resistance is on the rise.

Keywords: *Candida albicans*; Fluconazole resistance; *ERG11* overexpression; Droplet digital PCR; Phylogeny

1. Introduction

Candida albicans (*C. albicans*) is a dimorphic, opportunistic yeast that exists as part of the normal flora of the human gastrointestinal and genitourinary tracts. In healthy individuals, its proliferation is restricted by the host immune system and the balance of commensal microbiota. However, disturbances in this equilibrium, such as immunosuppression, hormonal fluctuations, antibiotic exposure, or changes in the vaginal microenvironment, can promote fungal overgrowth and infection (1). Among the diseases caused by this organism, vulvovaginal candidiasis (VVC) represents one of the most common mucosal fungal infections globally, affecting up to 75% of women at least once in their lifetime, with approximately 5% developing recurrent episodes (2, 3). Although not life-threatening, recurrent VVC (RVVC) substantially diminishes women's quality of life, reproductive health, and psychological well-being, often resulting in significant socioeconomic consequences due to repeated treatment costs and productivity loss.

The clinical burden of *C. albicans* infections is intensified by its diverse virulence traits, including morphological plasticity, biofilm formation, and the secretion of hydrolytic enzymes such as proteases, phospholipases, and lipases (4). These characteristics facilitate adherence, invasion, and evasion of host defences, while biofilm formation enhances antifungal tolerance and persistence on medical devices. The global increase in antifungal use, particularly azole-based therapies, has exerted selective pressure on *Candida* populations, resulting in the emergence of resistant phenotypes. This escalating resistance poses a critical challenge for clinical management, particularly in resource-limited settings where fluconazole remains the most accessible and commonly prescribed antifungal agent (5).

Azole antifungals, including fluconazole, itraconazole, and voriconazole, are widely regarded as first-line treatments for most *Candida* infections due to their broad spectrum of activity, low toxicity, and favourable pharmacokinetic profiles (6). Fluconazole is preferred due to its oral bioavailability, low cost, and proven efficacy in treating mucosal and systemic candidiasis. However, its extensive prophylactic and empirical use has contributed to the global rise in azole-resistant *C. albicans*, resulting in recurrent or persistent infections (7, 8). Resistance to fluconazole is complex and multifactorial, encompassing genetic, biochemical, and physiological adaptations that collectively reduce the susceptibility to the drug. These include point mutations in target enzymes, overexpression of efflux pumps, biofilm formation, and alterations in ergosterol biosynthesis (9, 10).

The principal molecular target of fluconazole is the lanosterol 14-α-demethylase (*ERG11*) gene, which encodes lanosterol 14-α-demethylase, a cytochrome P450-dependent enzyme essential in the biosynthetic pathway of ergosterol, the primary structural sterol of the fungal cell membrane. Inhibition of this enzyme disrupts the conversion of lanosterol to ergosterol, leading to the depletion of ergosterol and accumulation of toxic sterol intermediates that compromise membrane integrity and cellular viability (11). However, mutations or overexpression of the *ERG11* gene can restore enzyme function,

counteract fluconazole inhibition, and enable continued ergosterol synthesis, thereby promoting drug resistance (12, 13).

Multiple studies have reported a strong association between *ERG11* overexpression and azole resistance in *C. albicans* clinical isolates (14). Gain-of-function mutations in transcriptional regulators such as *UPC2* and *TAC1* further modulate resistance by enhancing the expression of *ERG11* and ABC transporters, including *CDR1* and *CDR2*, which actively expel fluconazole from the cell (15-17). This intricate regulatory interplay illustrates that azole resistance is rarely driven by a single mechanism but rather by a combination of transcriptional, structural, and physiological adaptations that allow the fungus to tolerate antifungal pressure (8, 18).

Despite extensive global research, a lack of molecular data persists regarding fluconazole resistance mechanisms in *C. albicans* isolates from Africa. In South Africa, fluconazole continues to be routinely prescribed for the treatment of candidiasis, particularly among immunocompromised patients (19, 20). Such frequent usage increases the likelihood of selective pressure and the development of resistance. Therefore, understanding the molecular basis of antifungal resistance within local isolates is crucial to inform therapeutic decision-making and antifungal stewardship initiatives (21).

The advent of molecular techniques has revolutionised the ability to characterise antifungal resistance mechanisms with high accuracy. While quantitative real-time polymerase chain reaction (qPCR) has long been employed for expression studies, droplet digital polymerase chain reaction (ddPCR) offers a more precise and sensitive approach. ddPCR allows for absolute quantification of nucleic acids by partitioning the sample into thousands of nanolitre-sized droplets, where each droplet serves as an individual amplification reaction (22, 23). This method reduces variability and eliminates the need for reference standard curves, allowing for reproducible detection of small yet biologically significant expression differences.

For accurate expression analysis, normalization against a stable housekeeping gene is essential. Actin-1 (*ACT1*), encoding actin, is a well-established reference gene for gene-expression studies using *Candida* species, providing a reliable baseline for relative quantification (24). By comparing *ERG11* expression relative to *ACT1*, it becomes possible to determine whether fluconazole resistance arises primarily through overexpression of the target gene or through alternative mechanisms, such as efflux pump activation or biofilm-associated tolerance (25).

In addition to gene-expression profiling, exploring sequence diversity among *ERG11* gene variants offers valuable insights into evolutionary adaptation and the development of resistance. Phylogenetic reconstruction using computational methods, such as the Neighbor-Joining (NJ) approach and the Maximum Composite Likelihood (MCL) model implemented in MEGA software (26), enables the assessment of genetic relatedness among resistant and susceptible isolates. Such analyses can reveal whether resistant isolates cluster within specific clades, potentially sharing conserved mutations

associated with antifungal tolerance. Combining expression data with phylogenetic analysis thus allows for a comprehensive understanding of resistance mechanisms and their evolutionary basis.

The present study was undertaken to investigate the molecular mechanisms underpinning fluconazole resistance in *C. albicans* clinical isolates by integrating phenotypic, molecular, and bioinformatic approaches. The study focuses on quantifying *ERG11* gene expression in fluconazole-resistant and susceptible isolates using ddPCR, with *ACT1* as the internal reference gene, and correlating these expression levels with antifungal susceptibility profiles. Furthermore, phylogenetic analysis of *ERG11* sequences was performed to evaluate genetic diversity and identify potential clustering patterns associated with resistance phenotypes.

This research is significant as it addresses a critical knowledge gap in the molecular epidemiology of azole resistance among *C. albicans* isolates from South Africa. By elucidating the transcriptional basis of fluconazole resistance, the study contributes to the global understanding of antifungal resistance mechanisms while providing region-specific data essential for future treatment guidelines. Ultimately, the insights gained from this work will support antifungal stewardship efforts, guide clinical decision-making, and contribute to improved patient outcomes in the management of *Candida* infections.

2. Methodology

2.1. Study Setting and Participant Population in the Parent Study

This laboratory-based study formed part of a larger research project aimed at identifying vaginitis and vaginosis pathogens in women (BREC/00003674/2021). In the main study, a total of 150 women were recruited from the Victoria Mxenge Hospital in Durban, KwaZulu-Natal, South Africa. Eligible participants were aged 18 years and older, provided written informed consent, and agreed to self-collect vaginal swab samples according to detailed instructions provided by the research team. Information on participants' sexual behaviour, medical history, and socio-demographic characteristics was obtained using a structured questionnaire administered by the study team. Recruitment took place between January and August 2022.

2.2. Ethical Approval for the Sub-Study

This sub-study received ethical clearance from the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (UKZN) under reference number BREC/00005995/2023.

2.3. Selection and Preparation of C. albicans Isolates

C. albicans isolates utilised in this study were sourced from the previous phase of the project, during which species identification and antifungal susceptibility testing had already been performed (27). From this collection, ten fluconazole-resistant isolates (ZMO10, ZMO11, ZMO35, ZMO44, ZMO65,

ZMO85, ZMO128, ZMO135, ZMO142, ZMO145) and one fluconazole-susceptible isolate (ZMO28) were selected for further investigation. All isolates were revived from glycerol stocks stored at -80°C and cultured on Sabouraud dextrose agar (SDA) supplemented with chloramphenicol (Neogen, United States). The plates were incubated aerobically at 37°C for 24 hours prior to experimental use.

2.4. Confirmatory Antifungal Susceptibility Assay for C. albicans Isolates

Susceptibility testing was conducted using the Sensititre™ YeastOne™ YO10 AST Plate (ThermoFisher Scientific, United States) to confirm the minimum inhibitory concentrations (MICs) of the eleven selected *C. albicans* isolates against fluconazole. An inoculum of *C. albicans* for each isolate was prepared to match a 0.5 McFarland standard. From this suspension, 20 µl was added to the Sensititre YeastOne Broth, followed by the addition of 100 µl of the inoculum into the microtiter plates. The plates were sealed and incubated at 35°C for 24 to 25 hours. All experiments were conducted in triplicate. Please refer to Table 1 for the MIC breakpoints, as per Clinical and Laboratory Standards Institute (CLSI) guidelines.

Table 1: MIC breakpoints as per CLSI guidelines.

Antifungal Agent	Susceptible	Susceptible-dose-dependent (SDD)	Resistant
Fluconazole	≤ 2 µg/ml	4 µg/ml	≥ 8 µg/ml

2.5. Ribonucleic acid (RNA) Extraction of Specific C. albicans Isolates

A total of eleven *C. albicans* isolates (ten fluconazole-resistant and one fluconazole-susceptible) were used for RNA extraction. Each isolate was cultured on SDA plates. Using a sterile inoculating loop, well-isolated colonies were aseptically transferred into sterile 1.5 ml RNase-free microcentrifuge tubes. Approximately 200-300 µl of sterile RNase-free water was added to each tube, and the suspensions were briefly vortexed to ensure even dispersal of the cells. Total RNA was extracted using the PureLink® RNA Mini Kit (ThermoFisher Scientific, Invitrogen, United States) according to the manufacturer's protocol for suspension cells. Briefly, cell suspensions were centrifuged at 2,000 × *g* for 5 minutes at 4°C, and the supernatant was discarded. Each pellet was resuspended in 600 µl Lysis Buffer containing 1% (v/v) 2-mercaptoethanol, followed by vigorous vortexing until complete dispersion was achieved.

Homogenisation was performed using a rotor-stator homogeniser at room temperature to ensure efficient cell disruption. To each homogenised lysate, one volume of 70% ethanol was added and mixed thoroughly. Up to 700 µl of the mixture was then loaded onto a PureLink® Spin Cartridge and centrifuged at 12,000 × *g* for 15 seconds at room temperature. The flow-through was discarded, and the loading process was repeated until the entire lysate had been processed. The columns were washed sequentially with 700 µl of Wash Buffer I, followed by two washes with 500 µl of Wash Buffer II

(containing ethanol), centrifuging at 12,000 × *g* for 15 seconds between each wash. After the final wash, the columns were centrifuged for an additional 1-2 minutes to remove residual ethanol. RNA was eluted in 30-100 µl of RNase-free water, incubated at room temperature for 1 minute, and centrifuged at 12,000 × *g* for 2 minutes into clean recovery tubes. The purified RNA was placed on ice for immediate use or stored at -80°C for long-term preservation. RNA concentration and purity were assessed spectrophotometrically at 260/280 nanometres, and RNA integrity was verified by agarose gel electrophoresis.

2.6. Complementary DNA (cDNA) Synthesis from the Total RNA of Specific C. albicans Isolates

cDNA was synthesised from total RNA using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, United States), following the manufacturer's instructions. For each reaction, 5 µl of total RNA was used as the template. Random Hexamer primers (1 µl) were employed to initiate cDNA synthesis. The total RNA, primer, and nuclease-free water were combined in a sterile, nuclease-free tube to a final volume of 12 µl. The mixture was briefly centrifuged, incubated at 65°C for 5 minutes to denature secondary structures, and then placed on ice prior to the addition of the remaining reagents. Subsequently, 4 µl of 5X Reaction Buffer, 1 µl of RiboLock RNase Inhibitor (20 U/µl), 2 µl of 10 mM dNTP Mix, and 1 µl of RevertAid M-MuLV Reverse Transcriptase (200 U/µl) were added to achieve a final reaction volume of 20 µl. The reaction mixture was gently mixed and briefly centrifuged, then incubated at 25°C for 5 minutes, followed by 60 minutes at 42°C to facilitate reverse transcription. The enzyme was inactivated by heating at 70°C for 5 minutes. The resulting cDNA was stored at -20°C for short-term use or at -70°C for long-term storage until required for downstream polymerase chain reaction (PCR) amplification and gene expression analyses.

2.7. ddPCR for Gene Expression

Gene expression was conducted on the one-plate digital PCR (dPCR) instrument (cat.no 911000 QIAcuity One, 2plex instrument), using the QIAcuity® EG (EvaGreen®) PCR Kit, 3× master mix (QIAGEN, Cat. No. 250111), and the QIAcuity Nanoplate 8.5K 96-well, (QIAGEN; Cat. No. 250021), with Nanoplate Seals (Cat. No. 250099) and Nanoplate Tray (Cat. No. 250098) as per manufacturer's instructions. The primer mix was made up of 4 µl EvaGreen® primer mix at 1.5 µl forward primer, 1.5 µl reverse primer, and 5 µl of cDNA, with a total reaction volume of 21 µl for each primer. dPCR conditions were as follows: Initial activation at 95ºC for 2 minutes, 35 cycles of Denaturation at 95ºC for 15 seconds, Annealing at 60ºC for 15 seconds, Extension at 72ºC for 15 seconds, and a single cooldown step at 40ºC for 5 minutes. Image settings were as per manufactures standard protocol for the initial imaging and were then adjusted for the second imaging to reduce saturation.

2.8. Data Analysis for Gene Expression

Target quantification was conducted using instrument software with well-specific volume precision factor applied for accurate concentration calculations. Absolute expression of the *ERG11* gene data was normalized to *ACT1*, and fold changes were calculated using a △△Ct method. Fold-change values greater than 1 were interpreted as upregulation, indicating increased gene expression; values less than 1 were classified as downregulation, representing decreased expression; and values approximately equal to 1 denoted no significant change in expression levels (Table 2). The *ERG11* expression primer sequences were as follows: Forward primer: 5'-ACCCTGAAGATTTTGATCCAACTAGATG-3', and reverse primer: 5'-CCCAAACCCATAATCAATTCATCAGA-3'. The *ACT1* expression primer sequences were as follows: 5'-GACAATTTCTCTTTCAGCACTAGTAGTG-3', and reverse primer: 5'-GCTGGTAGAGAGACTTGACCAACC-3' (14).

Table 2: Criteria for interpretation of gene expression regulation based on fold-change values.

Condition	Regulation
Fold Change > 1	Upregulated
Fold Change < 1	Downregulated
Fold Change ≈ 1	No change

2.8.1. Normalized Expression (△Ct) Calculation

Each sample's normalized expression was calculated by dividing its *ERG11* concentration by its *ACT1* concentration.

Normalized expression (△Ct) = *ERG11* (copies/µl) / *ACT1* (copies/µl)

2.8.2. Fold Change (△△Ct) and Log_2 Fold Change Calculations

Each sample's normalized expression was divided by the control normalized expression.

Fold change (△△Ct) = Normalized expression (fluconazole-resistant isolate) / Normalized Expression (ZMO28 (Control) isolate)

Log_2 Fold Change = $\log_2$(△△Ct)

2.9. DNA (deoxyribonucleic acid) Extraction

Genomic DNA was extracted from the eleven *C. albicans* isolates using the PureLink™ Microbiome DNA Purification Kit (ThermoFisher Scientific, United States) according to the manufacturer's instructions. The purified DNA samples were stored at -20°C until further use. The NanoDrop™ Spectrophotometer (ThermoFisher Scientific, United States) was used to measure the concentration and purity of the extracted DNA (27).

2.10. Amplification of the ERG11 Gene in C. albicans Isolates

PCR amplification of the *ERG11* gene was performed to confirm the presence and quality of the target gene prior to sequencing. The reaction mixture (25 µl total volume) comprised 12.5 µl of DreamTaq (2x) Master Mix (ThermoFisher Scientific, United States), 0.5 µl each of the forward and reverse primers, 1 µl of genomic DNA template, and 10.5 µl of nuclease-free water. The primer pair used for *ERG11* amplification was designed according to conserved regions of the *C. albicans ERG11* gene sequence: forward primer 5'-ACCCTGAAGATTTTGATCCAACTAGATG-3' and reverse primer 5'-CCCAAACCCATAATCAATTCATCAGA-3'. PCR reactions were performed in a BioRad Thermal Cycler under the following cycling conditions: initial denaturation at 95°C for 3 minutes; followed by 25 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute; with a final extension at 72°C for 5 minutes, and a hold at 4°C. The amplified products were electrophoresed on a 1% agarose gel and visualized using a gel documentation system. Successful amplification of *ERG11* yielded a single, distinct band of the expected product size, confirming DNA integrity and suitability for downstream sequencing analysis.

2.11. Sequencing of ERG11 PCR Amplicons

To confirm the identity of the amplified *ERG11* gene fragments and detect potential genetic variations associated with fluconazole resistance, Sanger sequencing was performed. The purified PCR amplicons were sent to Inqaba Biotechnological Industries (Pretoria, South Africa) for sequencing (one-direction), using an ABI 3500XL Genetic Analyzer (Applied Biosystems, USA). The resulting chromatograms were analysed and edited using Chromas software version 2.6.5 (Technelysium Pty Ltd., Queensland, Australia) to remove background noise and confirm base accuracy. The edited sequences were compared with reference *C. albicans ERG11* sequences available in the National Center for Biotechnology Information database using the Basic Local Alignment Search Tool to confirm gene identity.

2.12. Phylogenetic Analysis

Phylogenetic analysis of the *ERG11* gene sequences was performed to evaluate the evolutionary relationships among fluconazole-resistant and susceptible *C. albicans* isolates. The evolutionary history was inferred using the NJ method (28). The optimal tree with the sum of branch length = 0.243 is shown. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the MCL method (29), and were in the units of the number of base substitutions per site. The analytical procedure encompassed 10 coding nucleotide sequences using 1st, 2nd, 3rd, and non-coding positions. The pairwise deletion option was applied to all ambiguous positions for each sequence pair resulting in a final data set comprising 59 positions. Evolutionary analyses were conducted in MEGA12 (30).

3. Results

3.1. Confirmatory Antifungal Susceptibility Testing

The MICs of fluconazole for the *C. albicans* isolates ranged from 0.25 µg/ml to >256 µg/ml (Table 3). Among the eleven isolates tested, ten (90.9%) were classified as resistant to fluconazole, while only one isolate (ZMO28; 9.1%) was susceptible. The resistant isolates exhibited MIC values between 8 µg/ml and >256 µg/ml, with ZMO135 demonstrating the highest MIC value (>256 µg/ml). In contrast, ZMO28 showed an MIC of 0.25 µg/ml, indicating susceptibility to fluconazole according to Clinical and Laboratory Standards Institute (CLSI) breakpoints.

Table 3: MICs and susceptibility profiles of *C. albicans* isolates to fluconazole.

Isolate name	MIC (µg/ml)	Susceptibility profile
ZMO10	64	Resistant
ZMO11	32	Resistant
ZMO28	0.25	Susceptible
ZMO35	64	Resistant
ZMO44	32	Resistant
ZMO65	8	Resistant
ZMO85	8	Resistant
ZMO128	32	Resistant
ZMO135	> 256	Resistant
ZMO142	8	Resistant
ZMO145	16	Resistant

3.2. RNA Quantification of C. albicans Isolates

The total RNA concentrations extracted from the eleven *C. albicans* isolates varied notably, ranging from 4.30 ng/µl to 123.80 ng/µl (Table 4). Among the isolates, ZMO145 exhibited the highest RNA yield (123.80 ng/µl), followed by ZMO128 (88.90 ng/µl) and ZMO135 (74.90 ng/µl). In contrast, ZMO28 displayed the lowest RNA concentration (4.30 ng/µl). The variability in RNA concentrations observed across the isolates may reflect differences in initial yeast cell densities, extraction efficiencies, or physiological states of the isolates at the time of harvest. These RNA samples were subsequently used for downstream gene expression analysis.

Table 4: RNA concentrations (ng/µl) of *C. albicans* isolates obtained following total RNA extraction.

Isolate name	RNA concentrations (ng/µl)
ZMO10	4.70
ZMO11	6.40
ZMO28	4.30
ZMO35	30.90
ZMO44	26.20
ZMO65	33.80
ZMO85	60.80
ZMO128	88.90
ZMO135	74.90
ZMO142	59.30
ZMO145	123.80

3.3. cDNA Quantification of C. albicans Isolates

Following cDNA synthesis, all eleven *C. albicans* isolates yielded measurable concentrations suitable for downstream ddPCR analysis (Table 5). The cDNA concentrations ranged from 814.4 ng/µl to 2710.4 ng/µl. The lowest concentration was obtained from isolate ZMO10 (814.4 ng/µl), while the highest concentration was recorded for ZMO135 (2710.4 ng/µl). Most isolates demonstrated cDNA concentrations exceeding 2000 ng/µl, indicating efficient reverse transcription of extracted RNA and high nucleic acid integrity. The consistent amplification performance across isolates confirmed the suitability of the cDNA samples for accurate gene expression quantification.

Table 5: cDNA concentrations (ng/µl) of *C. albicans* isolates obtained following reverse transcription.

Isolate name	cDNA concentrations (ng/µl)
ZMO10	814.4
ZMO11	1744.2
ZMO28	1654.9
ZMO35	2009.1
ZMO44	2378.3
ZMO65	2558.1
ZMO85	2510.6
ZMO128	2614.3
ZMO135	2710.4
ZMO142	2467.7
ZMO145	2306.8

3.4. Quantitative Expression Analysis of the ERG11 Gene

The expression levels of the *ERG11* gene were quantified in fluconazole-resistant and -susceptible *C. albicans* isolates using ddPCR, with *ACT1* serving as the internal reference gene. The normalized expression values (*ERG11/ACT1*) were calculated, and fold-change differences were determined relative to the susceptible control isolate ZMO28 (set to 1.00). As shown in Table 6, ten isolates exhibited varying degrees of *ERG11* gene expression, with the majority showing transcriptional upregulation compared to the control. The normalized *ERG11* expression values ranged from 1.33 (ZMO128) to 33.41 (ZMO145). Nine isolates (ZMO10, ZMO11, ZMO35, ZMO44, ZMO65, ZMO85, ZMO135, ZMO142, and ZMO145) displayed fold-change values greater than 1.00, indicating *ERG11* upregulation, while only one isolate (ZMO128) showed downregulation (fold change = 0.15) (Figure 1). The highest *ERG11* expression was observed in isolates ZMO145 and ZMO65, which showed 3.73- and 3.68-fold increases relative to the control, with corresponding $\log_2$ fold-change values of 1.90 and 1.88, respectively. Similarly, isolates ZMO10, ZMO35, and ZMO135 exhibited strong upregulation with fold changes of 3.37, 2.85, and 3.28, respectively. Moderate increases were recorded in isolates ZMO11, ZMO44, ZMO85, and ZMO142, with fold-change values ranging between 1.31 and 2.78. In contrast, ZMO128 demonstrated a 0.15-fold decrease in *ERG11* expression ($\log_2$ = -2.76), suggesting potential involvement of alternative resistance mechanisms independent of *ERG11* regulation.

Table 6: Quantitative analysis of *ERG11* gene expression in *C. albicans* isolates determined by ddPCR.

Isolate name	*ERG11* (copies/µl)	*ACT1* (copies/µl)	Normalized expression (△Ct)	Fold change (△△Ct)	Log_2 fold change	Regulation
ZMO28 (Control)	4468.54	498.25	8.97	1.00	0.00	No change
ZMO10	3984.09	131.99	30.19	3.37	1.75	Upregulated
ZMO11	4360.54	371.33	11.74	1.31	0.39	Upregulated
ZMO35	4500.50	175.98	25.57	2.85	1.51	Upregulated
ZMO44	3836.81	220.43	17.41	1.95	0.96	Upregulated
ZMO65	4002.55	121.24	33.02	3.68	1.88	Upregulated
ZMO85	3959.64	158.86	24.93	2.78	1.48	Upregulated
ZMO128	10236.64	7713.22	1.33	0.15	-2.76	Downregulated
ZMO135	4428.12	150.59	29.40	3.28	1.71	Upregulated
ZMO142	4950.16	281.46	17.59	1.96	0.97	Upregulated
ZMO145	4547.96	136.16	33.41	3.73	1.90	Upregulated

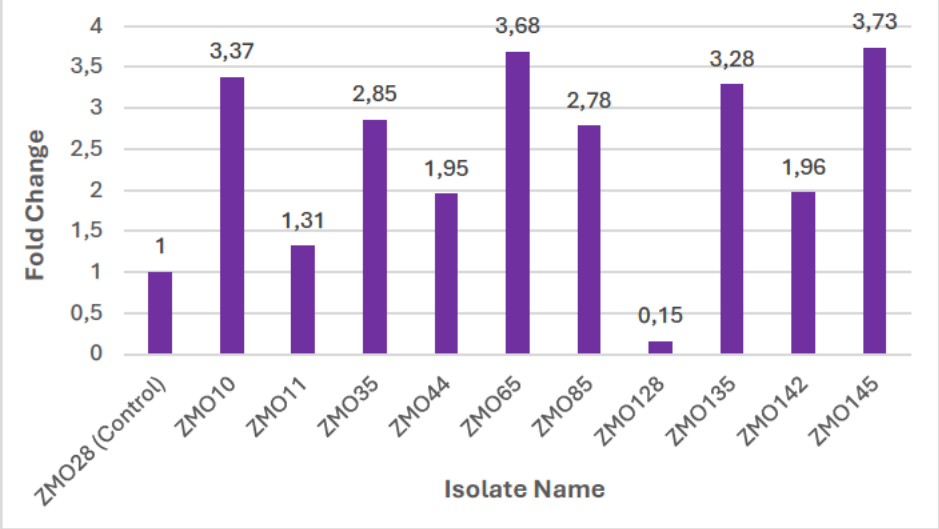

Figure 1: Relative *ERG11* gene expression in *C. albicans* isolates determined by ddPCR.

3.5. Quantification of ERG11 and ACT1 Gene Copy Numbers

Absolute quantification of *ERG11* and *ACT1* gene copy numbers was performed using ddPCR to determine gene expression levels across all *C. albicans* isolates. As illustrated in Figure 2, the *ERG11* gene exhibited consistently higher copy numbers compared to the housekeeping gene *ACT1* in all isolates. This indicates that *ERG11* was transcriptionally more active, particularly among fluconazole-

resistant isolates. Most isolates displayed *ERG11* copy numbers ranging between 3800 and 5000 copies/µl, while *ACT1* copy numbers remained markedly lower, between 120 and 500 copies/µl. Notably, isolate ZMO128 showed the highest *ERG11* and *ACT1* copy concentrations (10 236.64 copies/µl and 7713.22 copies/µl, respectively), suggesting an anomalous amplification pattern that differed from other resistant isolates. This result may reflect transcriptional variation, increased genomic content, or technical overexpression during amplification. When compared to the control isolate ZMO28, most resistant isolates (ZMO10, ZMO11, ZMO35, ZMO44, ZMO65, ZMO85, ZMO135, ZMO142, and ZMO145) demonstrated elevated *ERG11* copy numbers relative to *ACT1*, supporting the observation of *ERG11* upregulation as a possible mechanism contributing to fluconazole resistance (Table 6).

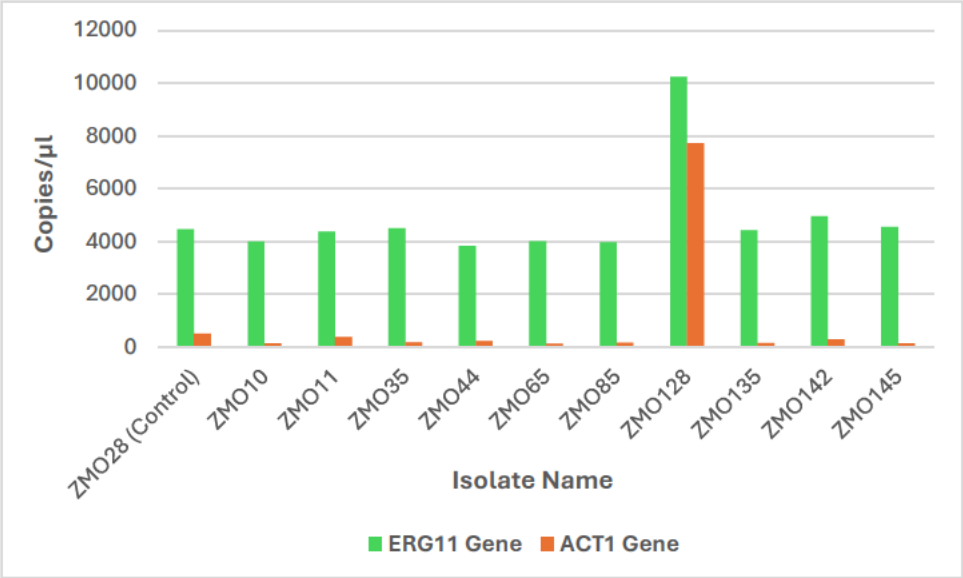

Figure 2: Absolute quantification of *ERG11* and *ACT1* gene copies in *C. albicans* isolates as determined by ddPCR.

3.6. Correlation between ERG11 Expression and Fluconazole Susceptibility

A comparative analysis was conducted to determine the relationship between *ERG11* gene expression and fluconazole MICs among the *C. albicans* isolates. As shown in Figure 3, isolates exhibiting higher *ERG11* fold-change expression (Figure 1) tended to display increased fluconazole MIC values (Table 3), indicating a positive association between *ERG11* overexpression and antifungal resistance. Notably, isolates ZMO145 and ZMO65 demonstrated the highest *ERG11* expression levels (3.73- and 3.68-fold, respectively), accompanied by moderate fluconazole MICs of 16 µg/ml and 8 µg/ml, respectively. However, isolates ZMO10 and ZMO135 exhibited strong upregulation of *ERG11* (>3-fold), correlating with the highest resistance levels (MICs ≥64 µg/ml). In contrast, the fluconazole-susceptible isolate

ZMO28 displayed the lowest MIC value of 0.25 µg/ml correlating with a reduced *ERG11* expression level (1.00-fold). Interestingly, ZMO128 exhibited the lowest *ERG11* expression level (0.15-fold) despite being phenotypically resistant with an elevated MIC of 32 µg/ml, suggesting that resistance in this isolate may be driven by alternative mechanisms such as efflux pump activation or biofilm-associated tolerance.

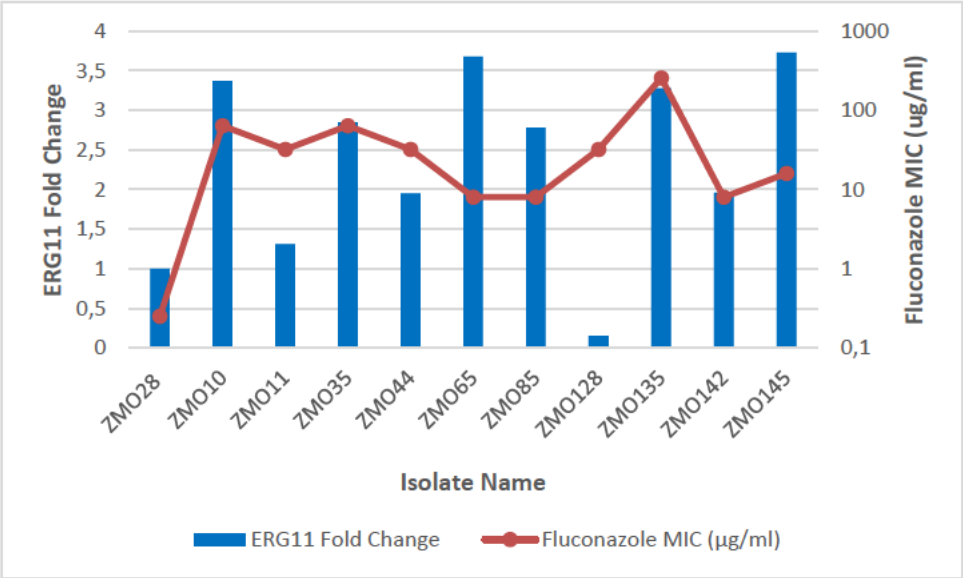

Figure 3: Correlation between *ERG11* gene expression (fold change) and fluconazole MICs among *C. albicans* isolates.

3.7. Phylogenetic Analysis of ERG11 Gene Sequences from C. albicans Isolates

Phylogenetic comparison of *ERG11* gene sequences among the ten fluconazole-resistant *C. albicans* isolates revealed distinct clustering patterns indicative of genetic diversity within the resistant population (Figure 4). The NJ tree, constructed using the MCL method in MEGA12, showed that nine isolates (ZMO10, ZMO11, ZMO35, ZMO44, ZMO65, ZMO85, ZMO135, ZMO142, and ZMO145) were grouped within a single major clade, indicating shared evolutionary ancestry and overall sequence similarity across the 59 analysed positions. Within this major clade, two internal sub-clusters were observed, reflecting possible sequence divergence and evolutionary adaptation associated with antifungal resistance. The first sub-cluster comprised isolates ZMO10, ZMO11, ZMO65, and ZMO145, which grouped closely together, suggesting a high degree of sequence similarity. Notably, ZMO10, ZMO65, and ZMO145 exhibited strong *ERG11* upregulation (>3-fold), and ZMO10, ZMO11, and ZMO145 showed elevated fluconazole MIC values ranging from 16 µg/ml to 64 µg/ml. Their close

genetic relatedness may indicate the presence of conserved point mutations within the *ERG11* coding region, particularly at loci known to alter lanosterol 14-α-demethylase structure and decrease fluconazole binding affinity. This phylogenetic grouping, therefore, aligns with both the phenotypic resistance profiles and gene expression data, supporting the hypothesis that shared sequence variants may contribute to enhanced azole tolerance.

The second sub-cluster included isolates ZMO35, ZMO44, ZMO85, ZMO135, and ZMO142. Although these isolates were also resistant to fluconazole, they displayed greater genetic variability, reflected by longer branch lengths within the cluster. This divergence may suggest the accumulation of independent mutations or recombination events within *ERG11* over time. Among these isolates, ZMO135, which demonstrated the highest MIC value (>256 µg/ml) together with marked *ERG11* upregulation (3.28-fold), may represent an evolutionary outlier with additional structural or regulatory modifications enhancing resistance intensity. In contrast, isolate ZMO128, despite being phenotypically resistant, occupied a separate and more distant branch from the main resistant clusters. This genetic distinctness, coupled with its markedly low *ERG11* expression (0.15-fold), suggests that resistance in ZMO128 may not be primarily driven by *ERG11* alterations, but rather by alternative mechanisms such as efflux pump activation or biofilm-mediated protection. ZMO28, the fluconazole-susceptible control, displayed basal placement on the tree, confirming its genetic divergence from the resistant isolates and its role as the ancestral reference within this dataset.

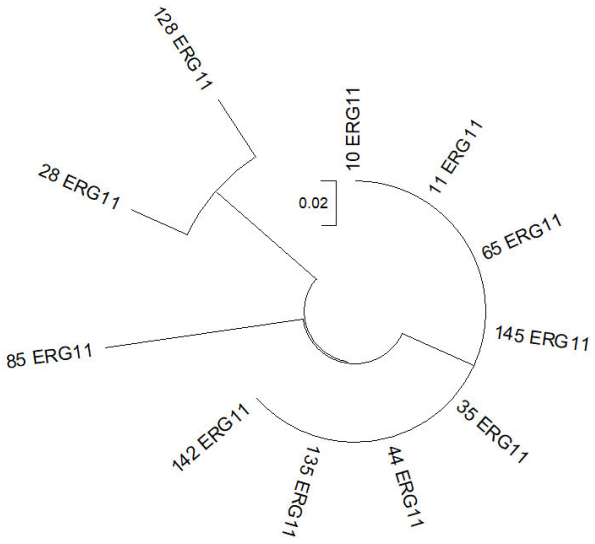

Figure 4: Phylogenetic relationships among fluconazole-resistant *C. albicans* isolates based on *ERG11* gene sequences.

4. Discussion

This study investigated the molecular and phenotypic basis of fluconazole resistance among *C. albicans* clinical isolates, focusing on *ERG11* gene expression and its relationship with antifungal susceptibility profiles. Several findings reflect a growing global concern, as azole resistance in *Candida* species has become increasingly prevalent in both hospital and community settings (8, 31). Fluconazole has long been a cornerstone antifungal agent due to its affordability, oral availability, and safety profile, particularly in low- and middle-income countries. However, its extensive and often empirical use has led to selection pressure that fosters resistant strains (6). In South Africa, fluconazole remains a first-line therapy for systemic and vulvovaginal candidiasis in immunocompromised populations, contributing to the growing burden of fluconazole-resistant *C. albicans* strains, leading to recurrent infections, prolonged therapy, and increased healthcare costs (19, 20).

The *ERG11* gene encodes lanosterol 14-α-demethylase, the target enzyme of azole antifungal agents, which catalyses a crucial step in the ergosterol biosynthesis pathway. Inhibition of this enzyme by fluconazole disrupts the synthesis of ergosterol, leading to membrane instability and fungal cell death (9). However, *ERG11* mutations and overexpression are well-established mechanisms that confer reduced drug binding affinity or compensate for inhibited enzyme activity (7, 32). In this study, ddPCR analysis demonstrated that nine of ten resistant isolates exhibited *ERG11* upregulation compared to the susceptible control (ZMO28). Fold-change values ranged from 1.31 to 3.73, confirming that transcriptional activation of *ERG11* plays a key role in mediating resistance. These results agree with previous investigations in Iran and Brazil, where *ERG11* overexpression was associated with elevated fluconazole MICs and persistent infections (14, 33).

Notably, isolates ZMO145 and ZMO65 exhibited the highest *ERG11* expression (3.73- and 3.68-fold), which correlated with MIC values of 16 µg/ml and 8 µg/ml, respectively. This supports earlier findings that *ERG11* overexpression contributes directly to decreased drug susceptibility by increasing the quantity of the target enzyme (34). However, high expression levels do not always correspond linearly with resistance intensity, suggesting that other regulatory elements influence expression outcomes. The positive correlation observed between *ERG11* fold-change values and fluconazole MICs suggests a direct functional relationship between gene expression and resistance phenotype. This mirrors findings from *Morais et al. (2021),* who reported that *ERG11* expression increases in tandem with rising MICs in clinical isolates subjected to chronic fluconazole exposure (35). Similarly, *Bhattacharya et al. (2020),* demonstrated that elevated *ERG11* transcript levels correspond with persistent infections and reduced therapeutic efficacy in immunocompromised patients (6).

The overexpression of *ERG11* is often regulated by upstream transcription factors, including *UPC2*, *TAC1*, and *MRR1*, which respond to cellular stress and antifungal exposure. *UPC2* functions as a sterol regulatory element-binding protein that activates genes involved in ergosterol synthesis, including

ERG11, upon ergosterol depletion (36). Mutations that enhance *UPC2* activity have been associated with constitutive *ERG11* overexpression and azole tolerance (16). Additionally, *TAC1* regulates ABC transporter genes (*CDR1* and *CDR2*), which mediate the efflux of azole drugs from fungal cells (17). Cross-talk between *ERG11* and efflux-related pathways results in combined resistance, where overexpression of both the target enzyme and efflux pumps amplify tolerance levels. Although efflux pump gene expression was not quantified in this study, the observed variability in *ERG11* fold-change values across isolates likely reflects differences in such regulatory mechanisms. Furthermore, *HSP90*- and calcineurin-mediated stress response pathways have been implicated in stabilising resistance phenotypes (37). *HSP90* facilitates proper folding of mutated *ERG11* proteins, enhancing their stability even under drug pressure, while calcineurin signalling contributes to cellular adaptation during membrane stress (38). The interplay among these molecular regulators underscores the complexity of antifungal resistance in *C. albicans*, suggesting that *ERG11* upregulation operates within a broader adaptive network rather than as an isolated event. Understanding this interplay will be a promising future study.

While *ERG11* overexpression was the dominant trend, isolate ZMO128 exhibited downregulation (0.15-fold) despite being phenotypically resistant (MIC = 32 µg/ml). This inconsistency highlights the multifactorial nature of fluconazole resistance, where resistance may occur through non-*ERG11*-mediated mechanisms. Efflux pump activation remains one of the most significant alternative pathways. Mutations in transcription factors (*TAC1* and *MRR1*), can trigger constitutive expression of efflux transporters (*CDR1*, *CDR2*, and *MDR1*), leading to active drug extrusion (39). *Teo et al. (2019)* reported *Candida* isolates exhibiting normal *ERG11* expression but high fluconazole MICs due to increased efflux activity (40). The downregulation of *ERG11* in ZMO128, therefore, may indicate that such efflux mechanisms compensate for decreased target gene expression. Biofilm formation represents another major contributor to antifungal tolerance. Biofilm-associated cells produce extracellular matrix components (β-glucans, mannans, and proteins) that limit drug penetration and create microenvironments conducive to stress adaptation (41). Moreover, biofilm-associated cells exhibit differential gene expression, including upregulation of *ALS*, *HWP1*, and *BCR1*, which reinforce adhesion and antifungal persistence (42). These alternative mechanisms might explain the resistance observed in isolate ZMO128, with low *ERG11* expression, supporting the concept of multifactorial resistance in *C. albicans*.

The phylogenetic analysis based on *ERG11* gene sequences revealed a single major resistant clade consisting of two internal sub-clusters, reflecting both conserved and divergent evolutionary patterns among resistant isolates. The first sub-cluster comprised of four isolates (ZMO10, ZMO11, ZMO65, and ZMO145), from this sub-cluster isolates ZMO10, ZMO65, and ZMO145 exhibited strong *ERG11* upregulation (>3-fold) and ZMO10, ZMO11, and ZMO145 showed elevated fluconazole MIC values ranging from 16 µg/ml to 64 µg/ml. Their clustering suggests a shared lineage or conserved point

mutations, possibly at codons Y132F or K143R, known to decrease fluconazole binding affinity (43, 44). The second sub-cluster comprised of five isolates (ZMO35, ZMO44, ZMO85, ZMO135, and ZMO142), which demonstrated greater genetic variability and moderate *ERG11* upregulation. Their longer branch lengths imply accumulation of independent mutations or recombination events that contribute to heterogeneity within the resistant population.

ZMO135, which showed the highest MIC (>256 µg/ml) together with marked *ERG11* upregulation (3.28-fold), may represent a hyper-resistant variant exhibiting both transcriptional and structural modifications in *ERG11*, consistent with findings by *Morais et al. (2021)* and *Li et al. (2025)* (35, 44). ZMO128 formed an independent branch, reflecting its unique genetic and transcriptional profile. Its divergence from other resistant isolates supports the hypothesis that fluconazole resistance can arise from multiple evolutionary routes, not all of which involve *ERG11*-mediated mechanisms. This phylogenetic diversity underscores the adaptive flexibility of *C. albicans* and its ability to evolve resistance through convergent molecular pathways (37). The clustering of highly expressed isolates within one phylogenetic group also implies selective conservation of *ERG11* regulatory elements under antifungal pressure. This phenomenon parallels findings by *Ren et al. (2025),* who reported that resistant clades often exhibit stabilising mutations enhancing enzyme affinity for endogenous substrates while reducing fluconazole binding (36). The phylogenetic data therefore provide an evolutionary framework linking molecular adaptation to phenotypic resistance.

The positive correlation between *ERG11* upregulation and high MICs suggests that molecular monitoring could improve clinical decision-making. Incorporating ddPCR-based assays into diagnostic workflows could allow early detection of resistant isolates before therapeutic failure occurs. The precision and sensitivity of ddPCR make it particularly suitable for low-resource settings, where culture-based susceptibility testing may be unavailable. At the epidemiological level, this study contributes novel insights into the molecular epidemiology of *C. albicans* in South Africa. Few local studies have combined phenotypic and genotypic analyses of resistance mechanisms, making this research a valuable reference point for future surveillance programs. The findings emphasise the importance of antifungal stewardship, restricting empirical fluconazole use and promoting culture-guided therapy to limit further resistance emergence. Furthermore, the identification of distinct phylogenetic clusters provides a molecular basis for tracking resistant strains within healthcare environments. Continuous monitoring of *ERG11* polymorphisms and expression patterns could inform infection control measures, helping prevent nosocomial transmission of resistant *Candida* lineages (6).

5. Limitations

This study provides important insights into *ERG11*-mediated fluconazole resistance; however, several limitations must be acknowledged. The sample size was small (n = 11), which restricts generalisability, although it allowed detailed molecular analysis. The study focused primarily on *ERG11* expression and sequence variation, even though azole resistance is multifactorial and may also involve efflux pump activation, transcriptional regulator mutations, and biofilm-associated tolerance. Lastly, the study did not perform protein-level verification of *ERG11* activity and additional phenotypic assays.

6. Conclusion

This study provides a comprehensive molecular assessment of fluconazole resistance in *C. albicans*, demonstrating that *ERG11* overexpression is a key driver of reduced susceptibility in most resistant isolates. The strong relationship between elevated *ERG11* expression levels, higher MICs, and the close phylogenetic clustering of resistant isolates highlights the importance of this gene in maintaining ergosterol biosynthesis under azole pressure. The divergence observed in isolates such as ZMO28 and ZMO128 further indicates that resistance may also arise from alternative, non-*ERG11*-dependent mechanisms, reinforcing the multifactorial nature of antifungal resistance. Going forward, broader genomic and transcriptomic profiling, including efflux pump genes, transcriptional regulators, and stress-response pathways, would provide deeper insight into resistance heterogeneity. Protein-level validation of *ERG11* activity and expanded sampling across clinical settings would strengthen future analyses. From a clinical perspective, incorporating routine molecular assays into diagnostic workflows may improve early detection of resistant strains, support targeted antifungal therapy, and guide stewardship strategies aimed at slowing the rise of azole resistance. Continued surveillance, coupled with advanced molecular tools, remains essential for improving patient outcomes and understanding the evolving epidemiology of *C. albicans* resistance.

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Conflict of Interest Disclosure

The authors declare no conflict of interest.

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References

1. Denning DW, Kneale M, Sobel JD, Rautemaa-Richardson R. Global burden of recurrent vulvovaginal candidiasis: a systematic review. The Lancet infectious diseases. 2018;18(11):e339-e47.
2. Nyirjesy P, Sobel JD. Vulvovaginal candidiasis. Obstet Gynecol Clin North Am. 2003;30(4):671-84.
3. Bitew A, Abebaw Y. Vulvovaginal candidiasis: species distribution of Candida and their antifungal susceptibility pattern. BMC women's health. 2018;18(1):94.
4. Calderone RA, Clancy CJ. Candida and candidiasis: American Society for Microbiology Press; 2011.
5. Pfaller MA, Diekema DJ, Turnidge JD, Castanheira M, Jones RN. Twenty Years of the SENTRY Antifungal Surveillance Program: Results for Candida Species From 1997-2016. Open Forum Infect Dis. 2019;6(Suppl 1):S79-s94.
6. Bhattacharya S, Sae-Tia S, Fries BC. Candidiasis and Mechanisms of Antifungal Resistance. Antibiotics (Basel). 2020;9(6).
7. Cowen LE, Sanglard D, Howard SJ, Rogers PD, Perlin DS. Mechanisms of antifungal drug resistance. Cold Spring Harbor perspectives in medicine. 2015;5(7):a019752.
8. Lee Y, Puumala E, Robbins N, Cowen LE. Antifungal drug resistance: molecular mechanisms in Candida albicans and beyond. Chemical reviews. 2020;121(6):3390-411.
9. Cernicka J, Subik J. Resistance mechanisms in fluconazole-resistant Candida albicans isolates from vaginal candidiasis. International journal of antimicrobial agents. 2006;27(5):403-8.

10. Nishimoto AT, Sharma C, Rogers PD. Molecular and genetic basis of azole antifungal resistance in the opportunistic pathogenic fungus Candida albicans. Journal of Antimicrobial Chemotherapy. 2019;75(2):257-70.
11. Rogers PD, Barker KS. Genome-Wide Expression Profile Analysis Reveals Coordinately Regulated Genes Associated with Stepwise Acquisition of Azole Resistance in Candida albicans Clinical Isolates. Antimicrobial Agents and Chemotherapy. 2003;47(4):1220-7.
12. de Oliveira Santos GC, Vasconcelos CC, Lopes AJO, de Sousa Cartágenes MdS, Filho AKDB, do Nascimento FRF, et al. Candida Infections and Therapeutic Strategies: Mechanisms of Action for Traditional and Alternative Agents. Frontiers in Microbiology. 2018;Volume 9 - 2018.
13. Esfahani A, Omran AN, Salehi Z, Shams-Ghahfarokhi M, Ghane M, Eybpoosh S, et al. Molecular epidemiology, antifungal susceptibility, and ERG11 gene mutation of Candida species isolated from vulvovaginal candidiasis: Comparison between recurrent and non-recurrent infections. Microbial Pathogenesis. 2022;170:105696.
14. Zare-Bidaki M, Maleki A, Ghanbarzadeh N, Nikoomanesh F. Expression pattern of drug-resistance genes ERG11 and TAC1 in Candida albicans Clinical isolates. Mol Biol Rep. 2022;49(12):11625-33.
15. Znaidi S, De Deken X, Weber S, Rigby T, Nantel A, Raymond M. The zinc cluster transcription factor Tac1p regulates PDR16 expression in Candida albicans. Molecular microbiology. 2007;66(2):440-52.
16. Flowers SA, Barker KS, Berkow EL, Toner G, Chadwick SG, Gygax SE, et al. Gain-of-function mutations in UPC2 are a frequent cause of ERG11 upregulation in azole-resistant clinical isolates of Candida albicans. Eukaryotic cell. 2012;11(10):1289-99.
17. Sasse C, Dunkel N, Schäfer T, Schneider S, Dierolf F, Ohlsen K, et al. The stepwise acquisition of fluconazole resistance mutations causes a gradual loss of fitness in Candida albicans. Molecular Microbiology. 2012;86(3):539-56.
18. Cowen LE, Steinbach WJ. Stress, Drugs, and Evolution: the Role of Cellular Signaling in Fungal Drug Resistance. Eukaryotic Cell. 2008;7(5):747-64.
19. Naicker SD, Govender N, Patel J, Zietsman IL, Wadula J, Coovadia Y, et al. Comparison of species-level identification and antifungal susceptibility results from diagnostic and reference laboratories for bloodstream Candida surveillance isolates, South Africa, 2009-2010. Sabouraudia. 2016;54(8):816-24.
20. Rabault C, Shuping L, Mpembe R, Quan V, Lanternier F, Lortholary O, et al. Recent Systemic Antifungal Exposure and Nonsusceptible Candida in Hospitalized Patients, South Africa, 2012–2017. Emerging Infectious Diseases. 2025;31(10):1901.
21. Chibabhai V. Incidence of candidemia and prevalence of azole-resistant candidemia at a tertiary South African hospital - A retrospective laboratory analysis 2016-2020. Southern African Journal of Infectious Diseases. 2022;37(1):326.

22. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Analytical chemistry. 2011;83(22):8604-10.
23. Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ, et al. Absolute quantification by droplet digital PCR versus analog real-time PCR. Nature methods. 2013;10(10):1003-5.
24. Paul S, Singh S, Chakrabarti A, Rudramurthy SM, Ghosh AK. Selection and evaluation of appropriate reference genes for RT-qPCR based expression analysis in Candida tropicalis following azole treatment. Scientific Reports. 2020;10(1):1972.
25. Pandey N, Tripathi M, Gupta MK, Tilak R. Overexpression of efflux pump transporter genes and mutations in ERG11 pave the way to fluconazole resistance in Candida tropicalis: a study from a North India region. Journal of Global Antimicrobial Resistance. 2020;22:374-8.
26. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular biology and evolution. 2016;33(7):1870-4.
27. Ramnarain C, Sukali G, Msomi N, Mabaso N, Molatlhegi RP, Abbai NS. Correlation between genotypes and antifungal susceptibility profiles of Candida isolates from pregnant and non-pregnant women in South Africa. The Journal of Medical Laboratory Science & Technology of South Africa. 2025;7(2):6-15.
28. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular biology and evolution. 1987;4(4):406-25.
29. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences. 2004;101(30):11030-5.
30. Kumar S, Stecher G, Suleski M, Sanderford M, Sharma S, Tamura K. MEGA12: Molecular Evolutionary Genetic Analysis version 12 for adaptive and green computing. Molecular Biology and Evolution. 2024;41(12):msae263.
31. Arastehfar A, Hilmioğlu-Polat S, Daneshnia F, Hafez A, Salehi M, Polat F, et al. Recent increase in the prevalence of fluconazole-non-susceptible Candida tropicalis blood isolates in Turkey: Clinical implication of azole-non-susceptible and fluconazole tolerant phenotypes and genotyping. Frontiers in microbiology. 2020;11:587278.
32. Bernaitis L, Devadharshini E, Sasmitha T, Pranavika A, Kalaivani K, Jaisharaba A. Mechanisms of Antifungal Resistance in Candida Species: Current Challenges and Future Directions. World Journal of Applied Medical Sciences. 2024;1(2):5-7.
33. Benedetti VP, Savi DC, Aluizio R, Adamoski D, Kava V, Galli-Terasawa LV, et al. ERG11 gene polymorphisms and susceptibility to fluconazole in Candida isolates from diabetic and kidney transplant patients. Revista da Sociedade Brasileira de Medicina Tropical. 2019;52:e20180473.

34. Flowers SA, Colón B, Whaley SG, Schuler MA, Rogers PD. Contribution of clinically derived mutations in ERG11 to azole resistance in Candida albicans. Antimicrob Agents Chemother. 2015;59(1):450-60.
35. Morais Vasconcelos Oliveira J, Conceição Oliver J, Latércia Tranches Dias A, Barbosa Padovan AC, Siqueira Caixeta E, Caixeta Franco Ariosa M. Detection of ERG11 Overexpression in Candida albicans isolates from environmental sources and clinical isolates treated with inhibitory and subinhibitory concentrations of fluconazole. Mycoses. 2021;64(2):220-7.
36. Ren Y, Zhu Q, Wu Y, Ju L, Liu J, Shen M, et al. Genomic Insights into Azole Resistance Mechanisms in Candida tropicalis Among Hematological Malignancy Patients with Candidemia. Current Microbiology. 2025;82(12):552.
37. Iyer KR, Robbins N, Cowen LE. The role of Candida albicans stress response pathways in antifungal tolerance and resistance. iScience. 2022;25(3).
38. Cowen LE. Hsp90 Orchestrates Stress Response Signaling Governing Fungal Drug Resistance. PLOS Pathogens. 2009;5(8):e1000471.
39. Arastehfar A, Lass-Flörl C, Garcia-Rubio R, Daneshnia F, Ilkit M, Boekhout T, et al. The Quiet and Underappreciated Rise of Drug-Resistant Invasive Fungal Pathogens. Journal of Fungi. 2020;6(3):138.
40. Teo JQ-M, Lee SJ-Y, Tan A-L, Lim RS-M, Cai Y, Lim T-P, et al. Molecular mechanisms of azole resistance in Candida bloodstream isolates. BMC Infectious Diseases. 2019;19(1):63.
41. Nett JE, Andes DR. Contributions of the biofilm matrix to Candida pathogenesis. Journal of Fungi. 2020;6(1):21.
42. Chong PP, Chin VK, Wong WF, Madhavan P, Yong VC, Looi CY. Transcriptomic and Genomic Approaches for Unravelling Candida albicans Biofilm Formation and Drug Resistance - An Update. Genes. 2018;9(11):540.
43. Ruiz-Baca E, Arredondo-Sánchez R, Corral-Pérez K, López-Rodríguez A, Meneses-Morales I, Ayala-Garcia V, et al. Molecular Mechanisms of Resistance to Antifungals in Candida albicans. 2021.
44. Li Y, Hind C, Furner-Pardoe J, Sutton JM, Rahman KM. Understanding the mechanisms of resistance to azole antifungals in Candida species. JAC-Antimicrobial Resistance. 2025;7(3).

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Chapter 3 revealed critical molecular determinants of fluconazole resistance, demonstrating that altered *ERG11* expression and phylogenetic relatedness contribute significantly to resistance phenotypes among the studied isolates. While these findings enhance understanding of resistance mechanisms, they also emphasise a pressing challenge: current antifungal therapies are increasingly inadequate against resistant and biofilm-forming *C. albicans*. The clinical implications of these resistance patterns highlight the urgent need for novel, accessible, and effective therapeutic alternatives. Building on this need, Chapter 4 evaluates the antifungal and antibiofilm potential of plant-based nanoemulsions and *Lactobacillus*-derived metabolites. This chapter examines whether natural therapeutic agents can inhibit planktonic growth, disrupt biofilms, and reduce virulence in resistant *C. albicans* isolates. By integrating traditional microbiology with innovative therapeutic approaches, Chapter 4 represents the applied component of this thesis and explores promising candidates for alternative or adjunctive treatment options in settings where fluconazole resistance is increasing.

CHAPTER 4

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Antibiofilm and antifungal activity of natural therapeutics against *Candida albicans*: A focus on plant nanoemulsions and *Lactobacillus* supernatants

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Abstract

Vulvovaginal candidiasis (VVC) is a common fungal infection predominantly caused by *Candida albicans* (*C. albicans*), with recurrence often linked to antifungal resistance and biofilm formation. Conventional azole therapy, including fluconazole, is increasingly undermined by resistant isolates, highlighting the need for alternative strategies. Plant-derived nanoemulsions and *Lactobacillus*-derived metabolites have emerged as promising candidates, yet their comparative antibiofilm effects against resistant isolates remain underexplored. This laboratory-based study evaluated the biofilm-forming capacity of 10 fluconazole-resistant *C. albicans* isolates and one fluconazole-susceptible *C. albicans* [American Type Culture Collection (ATCC)] 10231-strain. Biofilm inhibition was assessed using the microtiter plate (MTP) method following treatment with fluconazole, plant nanoemulsions prepared from *Ocimum tenuiflorum* (*O. tenuiflorum*), *Azadirachta indica* (*A. indica*), and *Moringa oleifera* (*M. oleifera*), and cell-free supernatants (CFSs) from four *Lactobacillus* strains: *Lactobacillus crispatus* (ATCC reference strain), *Lactobacillus reuteri* (isolated from a vaginal sample), *Lactobacillus delbrueckii*, and *Lactobacillus fermentum* (both isolated from plain yoghurt samples). All experiments were performed in triplicate, and results were expressed as mean optical density (492 nanometres) ± standard deviation. Statistical analysis was performed using a two-way analysis of variance, followed by Tukey's multiple comparisons test to assess differences between treatments across isolates. Fluconazole significantly reduced biofilm biomass in the fluconazole-susceptible ATCC 10231-strain but was only partially effective in resistant isolates, which retained strong biofilm phenotypes. In contrast, *O. tenuiflorum*, *A. indica*, and *M. oleifera* nanoemulsions completely suppressed biofilm formation at the undiluted concentration (1000 micromolar), shifting isolates into the non or weak category. The different *Lactobacillus* CFSs also inhibited biofilm development, though activity varied by strain and isolate. To our knowledge, this is the first study to directly compare plant nanoemulsions and *Lactobacillus* metabolites against both susceptible and resistant *C. albicans* isolates. These findings highlight the potential of these natural therapies as alternative strategies for managing resistant VVC.

Keywords: *Candida albicans*; Biofilms; Fluconazole; *Lactobacillus*; Nanoemulsions

1. Introduction

Candida species are among the most common fungal pathogens affecting humans, and they are capable of causing both superficial mucosal infections and life-threatening systemic diseases. Globally, vulvovaginal candidiasis (VVC) is one of the most frequent manifestations, with an estimated 70-75% of women experiencing at least one episode during their lifetime, and approximately 5-8% developing recurrent vulvovaginal candidiasis (RVVC), defined as four or more episodes within a year (1, 2). These infections not only reduce quality of life but also present economic burdens due to repeated medical visits and therapeutic interventions. The risk is heightened in immunocompromised individuals, pregnant women, and those with metabolic disorders such as diabetes mellitus, underscoring the importance of effective therapeutic strategies (3). *Candida albicans* (*C. albicans*) remains the predominant species associated with VVC; however, non-*albicans Candida* species such as *Candida glabrata*, *Candida tropicalis* (*C. tropicalis*), and *Candida parapsilosis* (*C. parapsilosis*) are increasingly isolated during VVC, some of which exhibit acquired resistance to standard antifungal drugs (4). The high recurrence rates and the emergence of resistant isolates highlight the limitations of current treatments and emphasize the urgent need for novel antifungal strategies.

The development of antifungal resistance is a multifactorial process driven by genomic and phenotypic adaptations. Resistance mechanisms involve mutations in drug target genes such as *ERG11*, which encodes lanosterol demethylase, the primary target of azoles. They also include the overexpression of efflux pumps (*CDR1*, *CDR2*, *MDR1*) and alterations in cell wall components targeted by echinocandins, such as *FKS1* or *FKS2* (5). Biofilm formation adds an additional layer of complexity. Biofilms are structured microbial communities encased in an extracellular matrix that protects embedded cells from host defences and antifungal penetration. *Candida* biofilms exhibit up to 1000-fold greater resistance to azoles compared to planktonic cells, and this makes them a central driver of persistent and recurrent infections (6).

Conventional antifungal therapies remain limited in their effectiveness against biofilm-associated infections. Azoles such as fluconazole are widely used due to their affordability and oral availability, however, resistance is increasingly reported, particularly in recurrent cases (7). Echinocandins, although effective against *C. albicans* biofilms, show reduced efficacy against *C. parapsilosis*, and resistance can develop via *FKS* mutations (7). Amphotericin B retains broad-spectrum activity but is hampered by nephrotoxicity and infusion-related side effects (8). Collectively, these limitations drive the search for alternative antifungal therapies that can overcome resistance and target biofilm-associated infections.

In recent years, there has been growing interest in plant-based and *Lactobacillus*-derived products as natural antifungal therapies. Plant extracts contain phytochemicals such as terpenoids, phenolics, flavonoids, and isothiocyanates that exhibit antifungal activity by disrupting cell membranes, inhibiting hyphal formation, and interfering with biofilm matrix production (9). However, crude extracts are often

limited by poor solubility, instability, and cytotoxicity at therapeutic doses. To overcome these barriers, nanoemulsion technology has emerged as a promising delivery platform. Nanoemulsions enhance solubility, improve bioavailability, and facilitate penetration into dense biofilms, leading to improved antifungal outcomes (10).

Ocimum tenuiflorum (*O. tenuiflorum*), *Azadirachta indica* (*A. indica*), and *Moringa oleifera* (*M. oleifera*) have long histories of use in traditional medicine and have demonstrated potent antimicrobial properties in modern investigations. Eugenol from *O. tenuiflorum* disrupts fungal membranes and suppresses biofilm-associated gene expression (11). *A. indica* contains limonoids and phenolics with documented antibiofilm activity, while *M. oleifera* produces isothiocyanates with fungicidal potential (12-14). Importantly, recent studies, including *Naicker et al. (2024)*, have confirmed that nanoemulsions prepared from these plants not only retain their antifungal activity but also demonstrate 0% haemolytic activity, highlighting their favourable safety profile (15).

Lactobacillus species also play an important role in maintaining vaginal health. By producing organic acids, hydrogen peroxide, and bacteriocins, they lower vaginal potential of hydrogen (pH), inhibit pathogen adhesion, and suppress filamentation in *Candida* species (16). Several studies have demonstrated that cell-free supernatants (CFSs) from *Lactobacillus rhamnosus* (*L. rhamnosus*), *Lactobacillus plantarum* (*L. plantarum*), and *Lactobacillus crispatus* (*L. crispatus*) inhibit *Candida* biofilms and reduce epithelial cell adhesion (17, 18). Moreover, *Lactobacillus*-derived biosurfactants have been shown to interfere with biofilm matrix formation, adding another mechanism of action (19). These properties make *Lactobacillus*-derived metabolites attractive as natural antifungal agents, particularly in the context of RVVC.

While many studies have examined natural antifungal agents, few have assessed the combined effects of *Lactobacillus*-derived metabolites and plant-derived compounds, or their effects on established *Candida* biofilms. RVVC remains a major challenge due to biofilm-associated resistance. This study evaluated the biofilm-forming ability of one fluconazole-susceptible *C. albicans* ATCC strain and 10 fluconazole-resistant *C. albicans* isolates. The antimicrobial and antibiofilm activities of *Lactobacillus* supernatants: *L. crispatus*, an ATCC reference strain, *Lactobacillus reuteri* (*L. reuteri*), which was isolated from a vaginal sample, *Lactobacillus delbrueckii* (*L. delbrueckii*), and *Lactobacillus fermentum* (*L. fermentum*), which were both isolated from yoghurt samples and nanoemulsions created from leaves of *O. tenuiflorum*, *A. indica*, and *M. oleifera* plants collected from the Botanical Gardens in Durban, South Africa, were tested, providing insights into their potential as natural alternatives for managing biofilm-related candidiasis.

2. Materials and Methods

2.1. Study Setting and Participant Population in the Parent Study

This laboratory-based study was a sub-study of a broader research project, which focused on diagnosing pathogens associated with vaginitis and vaginosis in women. In the parent study, 150 women were recruited from Victoria Mxenge Hospital in Durban, KwaZulu-Natal, South Africa. Participants in the main study were 18 years or older, provided written informed consent, and agreed to self-collect vaginal swabs, following sample collection instructions from the research team. Data on sexual behaviour, clinical history, and socio-demographic details were gathered from each participant through a structured questionnaire administered by the study team. The recruitment period for the study population spanned from January to August 2022 (BREC/00003674/2021).

2.2. Ethical Approval for the Sub-Study

Ethical approval for this sub-study was granted by the Biomedical Research Ethics Committee (BREC) at the University of KwaZulu-Natal (UKZN) under reference number (BREC/00005995/2023).

2.3. Selection and Preparation of C. albicans Isolates

C. albicans isolates used in this study were obtained from earlier phases of our project, where species identification and antifungal susceptibility testing had been performed (20). From this collection, ten fluconazole-resistant *C. albicans* isolates (ZMO10, ZMO11, ZMO35, ZMO44, ZMO65, ZMO85, ZMO128, ZMO135, ZMO142, ZMO145) and one fluconazole-susceptible reference strain (*C. albicans* ATCC 10231) were selected. Isolates were revived from glycerol stocks stored at -80°C and cultured on Sabouraud dextrose agar (SDA) plates containing chloramphenicol (Neogen, United States). The plates were then incubated aerobically at 37°C for 24 hours before experimentation.

2.4. Lactobacillus Strains and CFS Preparation

Four *Lactobacillus* strains were included in this study: *L. crispatus* (ATCC reference strain), *L. reuteri* (isolated from a vaginal sample), *L. delbrueckii*, and *L. fermentum* (both isolated from plain yoghurt samples), had been previously collected, isolated, and confirmed in our laboratory (unpublished data). For this study, strains were revived from frozen stocks in Man, Rogosa, and Sharpe (MRS) agar and incubated at 37°C for 72 hours under anaerobic conditions. The grown *Lactobacillus* cultures were then sub-cultured from the agar plates into 10 millilitres (ml) of MRS broth and incubated at 37°C for 48 hours under anaerobic conditions. After 48 hours, cultures were centrifuged at 12,000 revolutions per minute (rpm) for 10 minutes at 4°C to pellet the cells, and the supernatants were carefully harvested. The pH of each supernatant was adjusted to 7.5 using sterile sodium hydroxide, after which samples were sterilised by passage through 0.22 micrometre (μm) hydrophilic Durapore PVDF membranes

(Durapore® PVDF, Millipore, Burlington, United States). The pH-adjusted and filter-sterilised CFSs were then used for minimal inhibitory assays against *C. albicans* (21-23).

2.5. Broth Microdilution Assay for Assessing Antifungal Activity of Lactobacillus CFSs

The antifungal activity of crude *Lactobacillus* CFSs was evaluated using the broth microdilution method. Fifty microlitres (µl) of Sabouraud dextrose broth (SDB) was dispensed into columns 2-12 of sterile 96-well microtiter plates (MTPs). One hundred microlitres of crude *Lactobacillus* CFS was added to column 1, followed by serial two-fold dilutions across columns 2-11. The final 50 µl withdrawn from column 11 was discarded to ensure equal volumes in all wells. The *C. albicans* inoculum was prepared by transferring 1-2 colonies from fresh culture plates into 5 ml of sterile distilled water and mixing until the turbidity matched a 0.5 McFarland standard. One millilitre of this suspension was then added to 9 ml of SDB to yield the working inoculum. Fifty microlitres of inoculum was dispensed into each well (columns 1-12) using a multichannel pipette, beginning from column 12 and proceeding to column 1. Plates were sealed with parafilm and incubated at 37°C for 24 hours. All samples and controls were tested in triplicate. Crude CFSs of *L. crispatus, L. reuteri, L. delbrueckii,* and *L. fermentum* were tested against all fluconazole-resistant *C. albicans* isolates and the fluconazole-susceptible ATCC reference strain. Endpoint determination was performed visually using a minimum inhibitory concentration (MIC) mirror and by adding resazurin dye (0.02%). Wells that turned pink indicated *Candida* growth, while orange wells indicated inhibition. Because CFSs do not have a defined antifungal concentration, the minimum inhibitory factor (MIF) was recorded instead of the MIC. The MIF was defined as the lowest dilution of crude CFS that completely inhibited visible growth.

2.6. Collection and Preparation of Extracts from O. tenuiflorum, A. indica, and M. oleifera

O. tenuiflorum, A. indica, and *M. oleifera* leaves were collected from the Botanical Gardens in Durban, South Africa. After removing dust and debris, the leaves underwent an initial washing step by immersion in deionized water for 1 minute. The leaves were then left to air dry naturally for 4-5 days, shielded from direct sunlight, for the preparation of aqueous extracts. For extract preparation, the dried leaves were cut into small pieces using sterilized scissors, and approximately 100 grams (g) of leaf material was combined with 500 ml of distilled water and boiled for 30 minutes at 100°C. Following boiling, the mixture was allowed to cool and then filtered to obtain the aqueous extract. The extracts were further filtered using punched Whatman filter paper (No. 1) (Sigma-Aldrich, Germany) and subjected to centrifugation (1500 rpm for 10 minutes) to remove residual solid particles. The resulting extracts were stored at -20°C until use (15).

2.7. Nanoemulsion preparation from O. tenuiflorum, A. indica, and M. oleifera Extracts

The preparation of the oil phase involved creating a homogeneous organic solution comprising 400 µl of isopropyl myristate (Sigma-Aldrich, Germany) and 86 µl of Span 80 (Sigma-Aldrich, Germany), a lipophilic surfactant, dissolved in a water-miscible solvent. To prepare the aqueous phase, a homogeneous solution was formed by mixing 80 ml of water with 136 µl of Tween 80 (Sigma-Aldrich, Germany), a hydrophilic surfactant. Into this aqueous phase, 30 ml of the plant extract was introduced while the mixture was subjected to magnetic stirring. This resulted in the nearly instant formation of an oil-in-water emulsion as the organic solvent diffused into the external aqueous phase, creating nano-sized droplets. Magnetic stirring was continued for 30 minutes to allow the system to reach equilibrium. To remove the water-miscible solvent, the emulsion was subjected to evaporation for 45 minutes under reduced pressure while being centrifuged at 1000 rpm. Subsequently, the emulsion was cooled by immersing it in an ice bath for 10 minutes. Various nanoemulsion concentrations, namely 1000 micromolar (µM), 100 µM, 10 µM, and 1 µM, were prepared and stored at 4°C (15).

2.8. Evaluation of Antifungal Activity of Plant-Based Nanoemulsions

A disk diffusion method was adapted for this experiment. Overnight cultures of *Candida albicans* were grown on SDA, and a 0.5 McFarland standard was prepared using deionized water. Thereafter, 100 µl of the standardized inoculum was spread onto Mueller-Hinton agar plates. Sterile paper disks 6 millimetres (mm) were saturated with the nanoemulsions, with 50 µl of different concentrations (1000 µM, 100 µM, 10 µM, and 1 µM) added aseptically to each disk. The disks were then placed onto the Mueller-Hinton agar plates using aseptic techniques. Plates were incubated at 37°C for 24 hours under aerobic conditions. Antifungal activity was recorded as zones of clearance around the disks, and the diameters of these inhibition zones were measured in (mm) to determine inhibitory activity.

2.9. Quantitative Biofilm Formation Assay for C. albicans Isolates

The MTP assay described by *Millsap et al. (2001)* is the most widely used method and is considered the standard test for detecting biofilm formation (24). In the present study, ten fluconazole-resistant *Candida* isolates, along with the *Candida* ATCC strain, were tested for their ability to form biofilms. Individual wells of sterile, polystyrene, 96-well flat-bottom MTPs were filled with 100 µl aliquots of the *C. albicans* inoculum, and only SDB served as the control to check sterility and non-specific binding of the media. The MTPs were incubated aerobically for 72 hours at 37°C. After incubation, the contents of each well were gently removed by tapping the plates. The wells were washed four times with 200 µl of PBS to remove free-floating candidal cells. Biofilms formed by adherent candidal cells in the plate were stained with 100 µl of crystal violet (0.1%). Excess stain was rinsed off by thorough washing with 200 µl deionized water, and the plates were dried by inverting and air-drying aseptically at room temperature for approximately 30 minutes to ensure complete evaporation of residual moisture.

Adherent *Candida* cells formed a biofilm at the bottom of the wells and were uniformly stained with crystal violet. Optical density (OD) of stained adherent *Candida* biofilm was determined with a microplate reader using ADAP Plus software for analytical requirements (Biochrom Anthos, 2020) at a wavelength of 492 nanometres (nm). The OD values were considered as an index of *Candida* adhering to the surface and forming biofilms. Biofilm-forming capacity was classified as strong (>0.320), moderate (0.120-0.320), and non or weak (<0.120) (Table 1) (25).

Table 1: Classification of *Candida* adherence by the MTP method.

Mean OD values	Adherence	Biofilm formation
>0.320	Strong	High
0.120-0.320	Moderately	Moderate
<0.120	Non	Non or weak

2.10. Biofilm Inhibition of Plant Nanoemulsions & Lactobacillus CFSs Against C. albicans

The three different plant nanoemulsions (*O. tenuiflorum, A. indica, M. oleifera)* and four *Lactobacillus* supernatants (*L. crispatus, L. reuteri, L. delbrueckii,* and *L. fermentum*) were tested for their ability to inhibit biofilm formation of the ten fluconazole-resistant *Candida* isolates together with the *Candida* ATCC strain. Individual wells of sterile, polystyrene, 96-well flat-bottom MTPs were filled with 100 µl aliquots of the *C. albicans* inoculum and 100 µl of the specifically diluted plant nanoemulsion or *Lactobacillus* supernatant; only SDB served as the control. Biofilm formation and quantification were performed following the procedure described in section 2.9 (25).

2.11. Statistical Analysis of Treatment Effects on C. albicans Biofilm Inhibition

All experiments were performed in triplicate, and results are expressed as mean (OD 492 nm) ± standard deviation (SD). Statistical analysis was carried out using two-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test to assess differences between treatments across isolates. Separate analyses were performed for plant nanoemulsions (*O. tenuiflorum*, *A. indica*, and *M. oleifera*) and *Lactobacillus*-derived CFSs (*L. crispatus, L. reuteri, L. delbrueckii,* and *L. fermentum*) against the fluconazole-susceptible *C. albicans* ATCC strain and the fluconazole-resistant *C. albicans* isolates. An alpha level of 0.05 was applied to determine statistical significance, and all treatments demonstrated highly significant reductions in biofilm biomass compared to untreated controls ($p < 0.0001$). All analyses were performed using GraphPad Prism (Version 10.5.0; GraphPad Software, San Diego, USA).

3. Results

3.1. Broth Microdilution Assay for Assessing Antifungal Activity of Lactobacillus CFSs

The MIF of *L. crispatus* (*Lactobacillus* ATCC strain) against *C. albicans* isolates varied between the undiluted supernatant and dilutions up to 1:4. The fluconazole-susceptible *Candida* ATCC strain was inhibited at a dilution of 1:4 (Figure 1A). Among the resistant isolates, ZMO35 and ZMO65 were inhibited by a 1:2 MIF, while the majority, including ZMO10, ZMO11, ZMO44, ZMO85, ZMO135, and ZMO145, were inhibited only by a 1:1 MIF of *L. crispatus*. In contrast, isolates ZMO128 and ZMO142 (Figure 1B) required an undiluted supernatant for inhibition (Table 2).

As shown in Table 3, the MIF of *L. reuteri* against *C. albicans* isolates ranged from the undiluted supernatant to dilutions of 1:8. The fluconazole-susceptible *Candida* ATCC strain showed the highest susceptibility, with inhibition observed at a dilution of 1:8. Among the resistant isolates, ZMO11 (Figure 1C), ZMO44, and ZMO85 were inhibited at 1:2, while ZMO35 (Figure 1D)**,** ZMO65, ZMO128 and ZMO142 were inhibited at 1:1. In contrast, ZMO10, ZMO135, and ZMO145 required an undiluted supernatant for inhibition.

The MIF of *L. delbrueckii* against *C. albicans* isolates ranged from undiluted to 1:8. The fluconazole-susceptible *Candida* ATCC strain was the most susceptible, showing inhibition at a dilution of 1:8. Among the resistant isolates, ZMO11 (Figure 1C), ZMO44, and ZMO85 were inhibited at 1:2, while ZMO35 (Figure 1D), ZMO65, ZMO128, and ZMO142 were inhibited at 1:1. In contrast, ZMO10, ZMO135, and ZMO145 required an undiluted supernatant for inhibition, suggesting reduced susceptibility (Table 4).

As shown in Table 5, the MIF of *L. fermentum* against *C. albicans* isolates ranged from undiluted to 1:4. The fluconazole-susceptible *Candida* ATCC strain showed inhibition at a dilution factor of 1:4 (Figure 1A). Among the resistant isolates, ZMO35 and ZMO65 were inhibited at a 1:2 dilution factor, while the majority, including ZMO10, ZMO11, ZMO44, ZMO85, ZMO135, and ZMO145, required a 1:1 dilution for inhibition. In contrast, ZMO128 and ZMO142 (Figure 1B) were inhibited only by the undiluted supernatant, demonstrating reduced susceptibility.

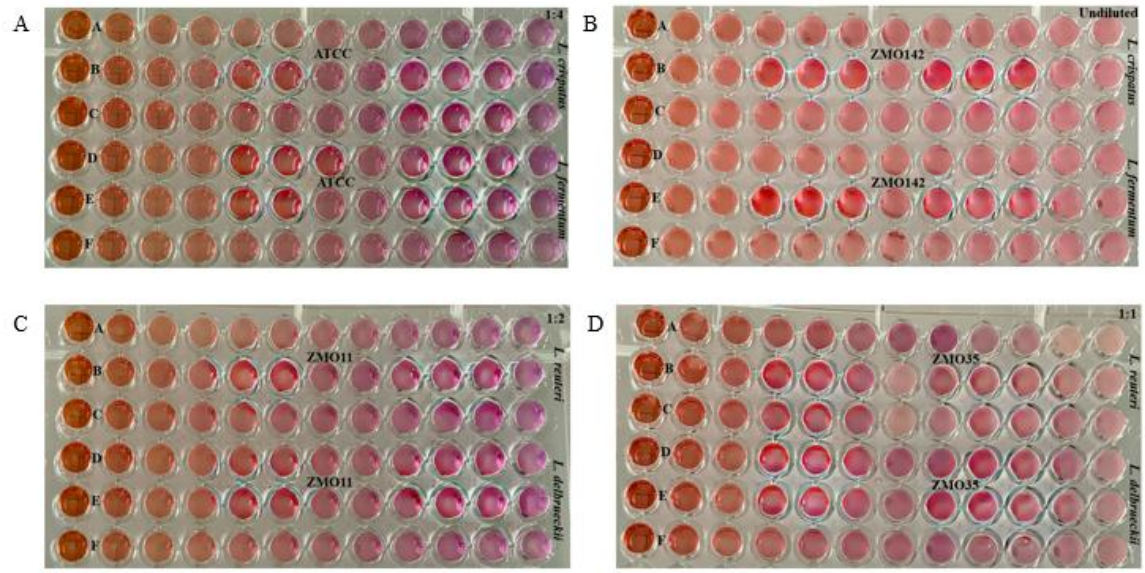

Figure 1: MIF determination of *Lactobacillus* strains against clinical and ATCC strains of *C. albicans*, with (A) showing the effect of *L. crispatus* (rows A, B, and C) and *L. fermentum* (rows D, E, and F) against the fluconazole-susceptible *C. albicans* ATCC strain. (B) is MIF determination of *L. crispatus* (rows A, B, and C) and *L. fermentum* (rows D, E, and F) against *C. albicans* isolate ZMO142; while (C) is the effect of *L. reuteri* (rows A, B, and C) and *L. delbrueckii* (rows D, E, and F) against the fluconazole-resistant *C. albicans* isolate ZMO11. Lastly, (D) is the MIF determination of *L. reuteri* (rows A, B, and C) and *L. delbrueckii* (rows D, E, and F) against the fluconazole-resistant *C. albicans* isolate ZMO35. The pink colour denotes *C. albicans* growth and the orange colour shows growth inhibition.

Table 2: MIF of *L. crispatus* against the fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates (undiluted to 1:4).

Isolate Name	MIF (*L. Crispatus*)
ATCC	1:4
ZMO10	1:1
ZMO11	1:1
ZMO35	1:2
ZMO44	1:1
ZMO65	1:2
ZMO85	1:1
ZMO128	Undiluted
ZMO135	1:1
ZMO142	Undiluted
ZMO145	1:1

Table 3: MIF of *L. reuteri* against the fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates (undiluted to 1:8).

Isolate Name	MIF (*L. reuteri*)
ATCC	1:8
ZMO10	Undiluted
ZMO11	1:2
ZMO35	1:1
ZMO44	1:2
ZMO65	1:1
ZMO85	1:2
ZMO128	1:1
ZMO135	Undiluted
ZMO142	1:1
ZMO145	Undiluted

Table 4: MIF of *L. delbrueckii* against the fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates (undiluted to 1:8).

Isolate Name	MIF (*L. delbrueckii*)
ATCC	1:8
ZMO10	Undiluted
ZMO11	1:2
ZMO35	1:1
ZMO44	1:2
ZMO65	1:1
ZMO85	1:2
ZMO128	1:1
ZMO135	Undiluted
ZMO142	1:1
ZMO145	Undiluted

Table 5: MIF of *L. fermentum* against the fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates (undiluted to 1:4).

Isolate Name	MIF (*L. fermentum*)
ATCC	1:4
ZMO10	1:1
ZMO11	1:1
ZMO35	1:2
ZMO44	1:1
ZMO65	1:2
ZMO85	1:1
ZMO128	Undiluted
ZMO135	1:1
ZMO142	Undiluted
ZMO145	1:1

3.2. Evaluation of Antimicrobial Activity of Plant-Based Nanoemulsions

Supplementary Table 1 shows the inhibitory effect of the *O. tenuiflorum* nanoemulsion against the fluconazole-susceptible *C. albicans* ATCC strain, and the ten fluconazole-resistant *C. albicans* isolates were evaluated at concentrations ranging from 1000 µM to 1 µM. Complete inhibition (no growth) was observed at 1000 µM across all isolates tested, including the fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates ZMO10-ZMO145 (Figure 2). At lower concentrations (100 µM, 10 µM, and 1 µM), growth was observed in all isolates, indicating a concentration-dependent antifungal effect where only the undiluted preparation was effective in suppressing *Candida* growth.

As shown in Table 6, the antifungal activity of the *O. tenuiflorum* nanoemulsion was assessed using the agar well diffusion method across the fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates. The zones of inhibition ranged from 16-18 mm, indicating consistent antifungal effects. The largest inhibition zones were observed against the ATCC strain (Figure 2) and isolate ZMO11 (18 mm), followed by ZMO10 and ZMO135 (17 mm). All other isolates, including ZMO35, ZMO44, ZMO65, ZMO85, ZMO128, ZMO142, and ZMO145 (Figure 2), demonstrated inhibition zones of 16 mm.

Supplementary Table 2 shows the inhibitory effect of the *A. indica* nanoemulsion against the fluconazole-susceptible *C. albicans* ATCC strain, and the ten fluconazole-resistant *C. albicans* isolates were evaluated at concentrations ranging from 1000 µM to 1 µM. Complete inhibition (no growth) was observed at 1000 µM across all isolates, including the fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates ZMO10-ZMO145 (Figure 3). At lower concentrations (100 µM, 10 µM, and 1 µM), growth was detected in all isolates, indicating that the *A. indica* nanoemulsion exhibits a concentration-dependent antifungal effect, with complete suppression of *Candida* growth only at the highest concentration tested.

As shown in Table 7, the antifungal potential of the *A. indica* nanoemulsion was evaluated against the fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates. The inhibition zones ranged from 17-20 mm, reflecting strong and consistent antifungal activity. The largest inhibition zones (20 mm) were observed in the ATCC strain (Figure 3) and isolate ZMO128, followed closely by ZMO11 and ZMO142 (19 mm). Several isolates, including ZMO35, ZMO44 (Figure 3), ZMO85, and ZMO145, showed inhibition zones of 18 mm, while ZMO10, ZMO65, and ZMO135 demonstrated slightly smaller zones of 17 mm.

Supplementary Table 3 shows the inhibitory effect of the *M. oleifera* nanoemulsion against the fluconazole-susceptible *C. albicans* ATCC strain, and the ten fluconazole-resistant *C. albicans* isolates

were evaluated at concentrations ranging from 1000 µM to 1 µM. Complete inhibition (no growth) was observed at 1000 µM across all isolates, including the fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates ZMO10-ZMO145 (Figure 4). At lower concentrations (100 µM, 10 µM, and 1 µM), growth was consistently observed in all isolates, indicating that the *M. oleifera* nanoemulsion exerts a concentration-dependent antifungal effect, with full suppression of *Candida* growth only at the highest concentration tested.

As shown in Table 8, the antifungal activity of the *M. oleifera* nanoemulsion demonstrated the strongest inhibition zones among the three plant extracts, with values ranging from 19-22 mm across the fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates. The largest inhibition zones were observed in ATCC (Figure 4) and ZMO11 (22 mm), followed closely by ZMO10, ZMO35, ZMO65, and ZMO145 (21 mm). Inhibition zones of 20 mm were recorded in ZMO44, ZMO85, ZMO135, and ZMO142, while ZMO128 (Figure 4) showed the lowest zone of inhibition at 19 mm.

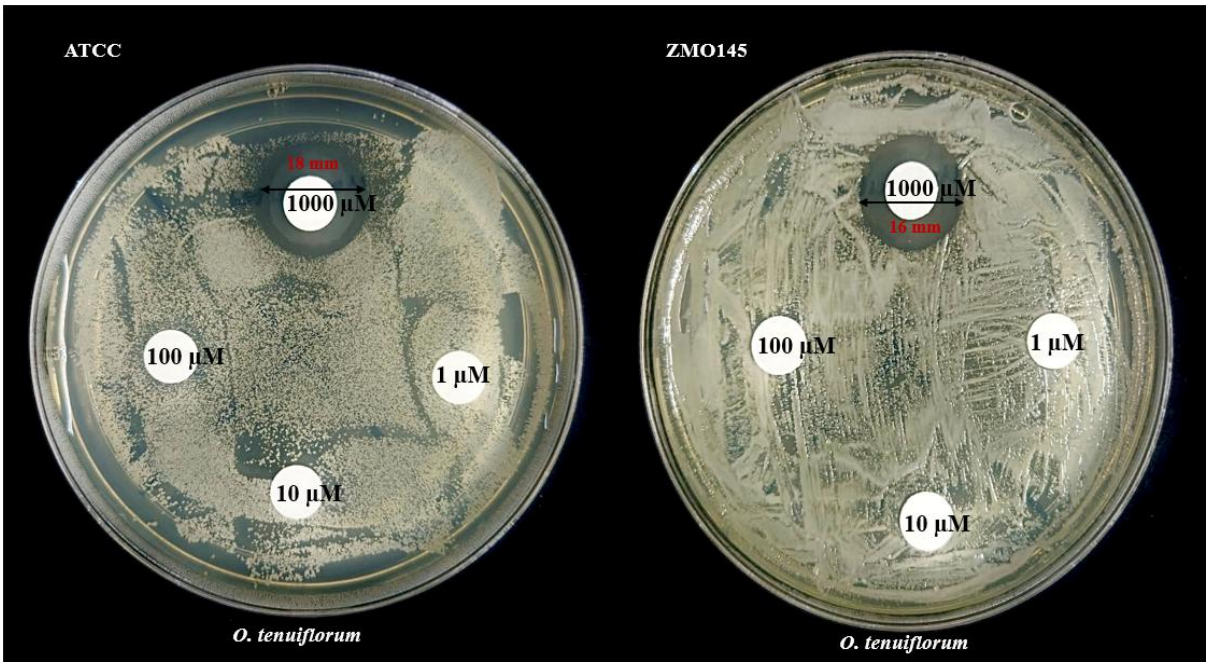

Figure 2: Antifungal activity of the *O. tenuiflorum* nanoemulsion against the *C. albicans* ATCC fluconazole-susceptible strain with an inhibition zone of 18 mm (left) and the ZMO145 fluconazole-resistant *C. albicans* isolate with an inhibition zone of 16 mm (right). Clear zones of inhibition were observed at 1000 µM, while no inhibition was detected at lower concentrations (100 µM, 10 µM, and 1 µM).

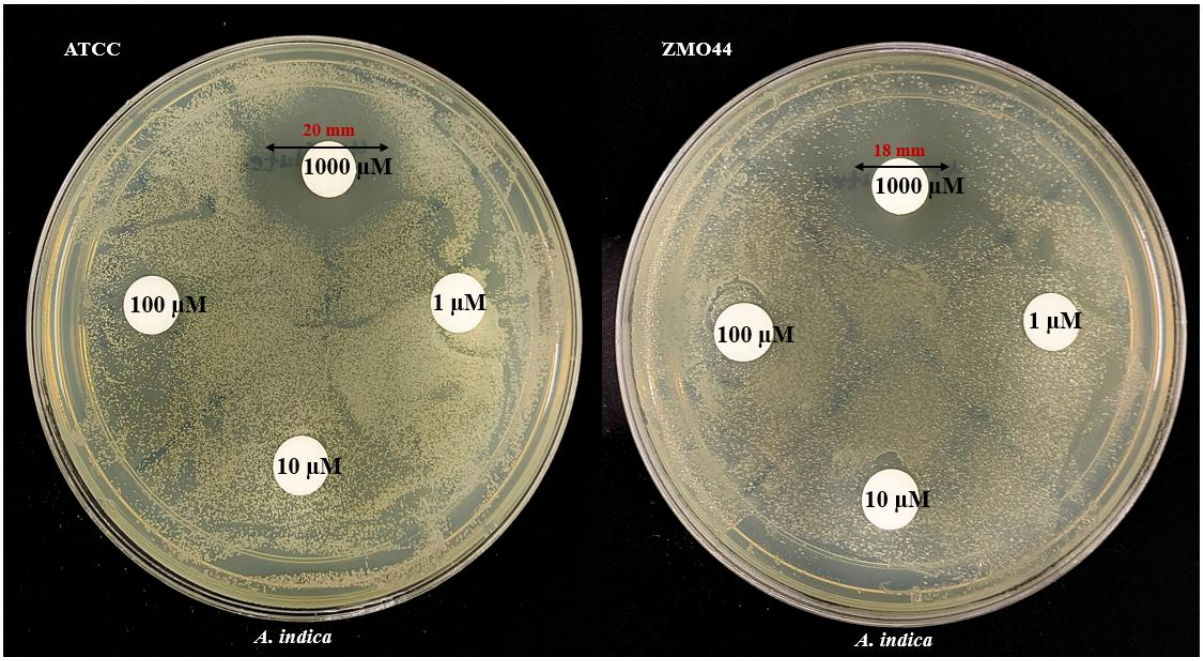

Figure 3: Antifungal activity of the *A. indica* nanoemulsion against the fluconazole-susceptible *C. albicans* ATCC strain with an inhibition zone of 20 mm (left) and the fluconazole-resistant ZMO44 isolate with an inhibition zone of 18 mm (right). Growth inhibition was observed at 1000 µM, while no inhibition was seen at 100 µM, 10 µM, and 1 µM.

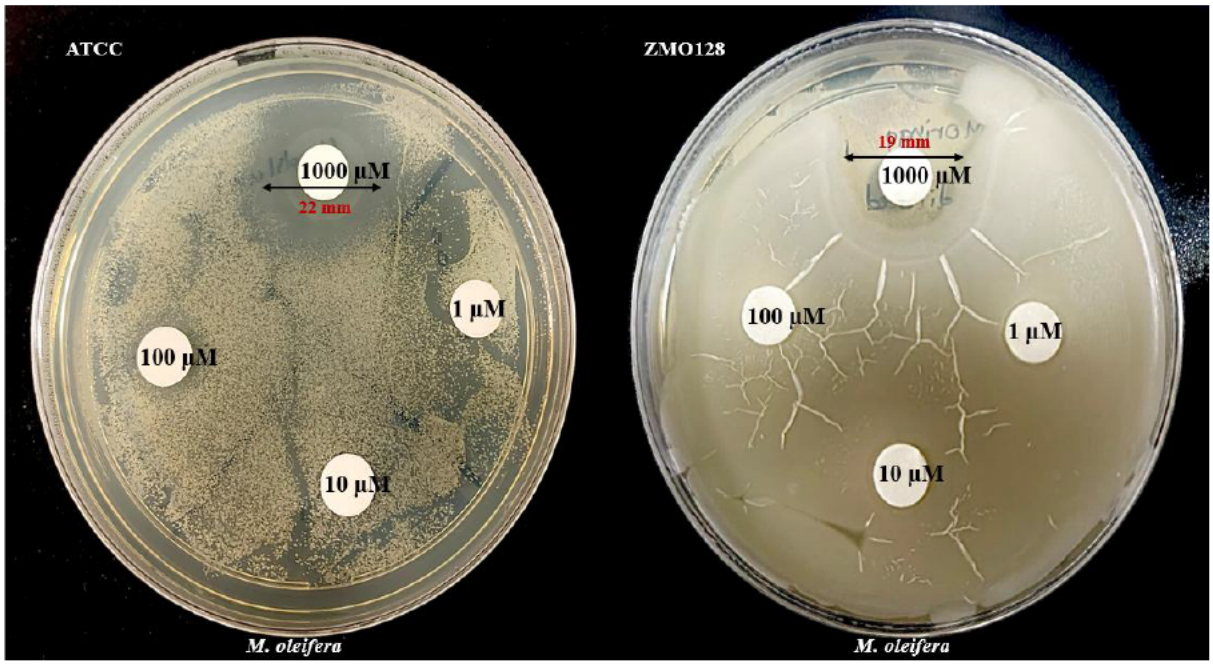

Figure 4: Antifungal activity of the *M. oleifera* nanoemulsion against the fluconazole-susceptible *C. albicans* ATCC strain with an inhibition zone of 22 mm (left) and the fluconazole-resistant ZMO128 *C. albicans* isolate with an inhibition zone of 19 mm (right). Growth inhibition was observed at 1000 µM, while no inhibition was detected at 100 µM, 10 µM, or 1 µM.

Table 6: Antifungal activity of the *O. tenuiflorum* nanoemulsion against the fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates, expressed as average zones of inhibition (mm).

Isolate Name	*O. tenuiflorum* (Average Zone in mm)
ATCC	18 mm
ZMO10	17 mm
ZMO11	18 mm
ZMO35	16 mm
ZMO44	16 mm
ZMO65	16 mm
ZMO85	16 mm
ZMO128	16 mm
ZMO135	17 mm
ZMO142	16 mm
ZMO145	16 mm

Table 7: Antifungal activity of the *A. indica* nanoemulsion against the *C. albicans* ATCC fluconazole-susceptible strain and the ten fluconazole-resistant *C. albicans* isolates, expressed as average zones of inhibition (mm).

Isolate Name	*A. indica* (Average Zone in mm)
ATCC	20 mm
ZMO10	17 mm
ZMO11	19 mm
ZMO35	18 mm
ZMO44	18 mm
ZMO65	17 mm
ZMO85	18 mm
ZMO128	20 mm
ZMO135	17 mm
ZMO142	19 mm
ZMO145	18 mm

Table 8: Antifungal activity of the *M. oleifera* nanoemulsion against the *C. albicans* fluconazole-susceptible ATCC strain and the ten fluconazole-resistant *C. albicans* isolates, expressed as average zones of inhibition (mm).

Isolate Name	*M. oleifera* (Average Zone in mm)
ATCC	22 mm
ZMO10	21 mm
ZMO11	22 mm
ZMO35	21 mm
ZMO44	20 mm
ZMO65	21 mm
ZMO85	20 mm
ZMO128	19 mm
ZMO135	20 mm
ZMO142	20 mm
ZMO145	21 mm

3.3. Quantitative Biofilm Formation Assay for C. albicans Isolates

Biofilm formation by the fluconazole-susceptible *C. albicans* ATCC strain and ten fluconazole-resistant *C. albicans* isolates was assessed using the MTP assay (Figure 5). As expected, the negative control wells (A1-A3), containing SDB only, showed no visible biofilm formation. In contrast, the fluconazole-susceptible *C. albicans* ATCC strain (A4-A6) and all ten fluconazole-resistant *C. albicans* isolates (ZMO10-ZMO145) demonstrated visible biofilm development to varying extents, confirming the ability of these *Candida* isolates to form structured biofilms under *in vitro* conditions. This result established the baseline adherence and growth characteristics of the tested isolates, providing a platform for subsequent evaluation of antifungal and inhibitory treatments.

Crystal violet staining of biofilms confirmed robust biofilm formation by the fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates (Figure 6). Negative control wells (A1-A3), containing SDB alone, showed no staining, confirming the absence of biofilms. In contrast, all isolates demonstrated visible staining intensity, consistent with the presence of adherent biofilm biomass. The fluconazole-susceptible *C. albicans* ATCC strain (A4-A6) and the ten fluconazole-resistant *C. albicans* isolates (ZMO10-ZMO145) all exhibited marked crystal violet retention, though qualitative differences in staining intensity suggested variability in the strength of biofilm formation between isolates. This visualization further validated the adherence and biomass production of the tested isolates, serving as a baseline for subsequent quantitative OD measurements and treatment comparisons.

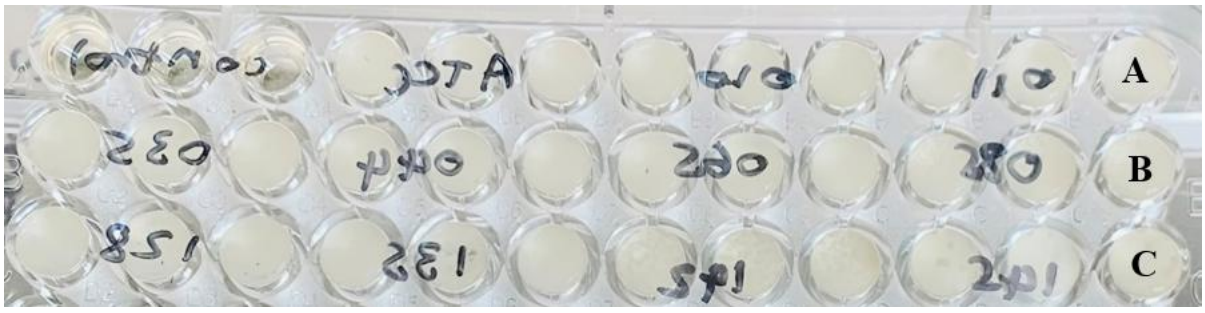

Figure 5: Biofilm formation of the fluconazole-susceptible *C. albicans* ATCC strain and ten fluconazole-resistant *C. albicans* isolates (ZMO10-ZMO145) using the MTP assay. Negative control wells (A1-A3) contained SDB only, showing no biofilm formation. Visible biofilms were observed in all *C. albicans* isolates tested.

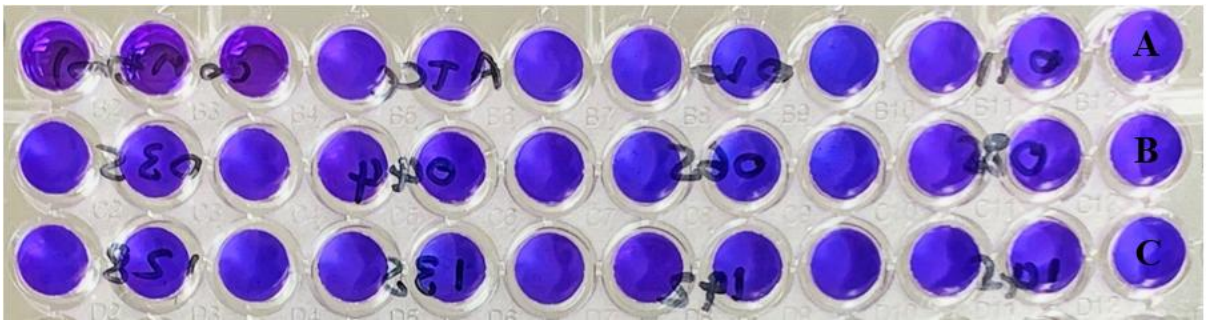

Figure 6: Crystal violet staining of biofilms formed by the fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates (ZMO10-ZMO145) using the MTP assay. Negative control wells (A1-A3) contained SDB only, showing no staining. Visible staining in all other wells confirmed the production of biofilm biomass.

3.4. Biofilm Inhibition of Plant Nanoemulsions & Lactobacillus CFSs Against C. albicans

As shown in Figure 7 and Table 9, untreated negative controls exhibited strong biofilm formation across all *C. albicans* isolates, with OD values ranging from 0.543 ± 0.024 (ATCC strain) to 1.060 ± 0.010 (ZMO10 isolate). According to the MTP classification system, these values correspond to strong adherence and high biofilm formation, confirming the robust biofilm-forming capacity of the isolates under baseline conditions.

Treatment with fluconazole significantly reduced biofilm biomass ($p < 0.0001$). The fluconazole-susceptible (ATCC strain) decreased to 0.067 ± 0.009, falling into the non or weak category, whereas the fluconazole-resistant isolates showed OD values between 0.355 ± 0.018 (ZMO135 isolate) and 0.385 ± 0.012 (ZMO128 isolate). Despite reductions compared to the negative controls, the resistant isolates remained above the >0.320 threshold, confirming persistent strong biofilm formation when treated with fluconazole.

In contrast, *O. tenuiflorum*, *A. indica*, and *M. oleifera* nanoemulsions exerted more profound and consistent effects on biofilm inhibition ($p < 0.0001$). Across all *C. albicans* isolates, OD values were reduced below the <0.120 threshold. For *O. tenuiflorum*, values ranged from 0.083 ± 0.002 (ATCC strain) to 0.117 ± 0.002 (ZMO135 isolate); for *A. indica*, from 0.086 ± 0.003 (ATCC strain) to 0.117 ± 0.001 (ZMO35 isolate); and for *M. oleifera*, from 0.069 ± 0.002 (ATCC strain) to 0.111 ± 0.006 (ZMO35 isolate). These results demonstrated that the plant-derived nanoemulsions completely inhibited biofilm development in both the fluconazole-susceptible *C. albicans* ATCC strain and the fluconazole-resistant *C. albicans* isolates (ZMO10, ZMO11, ZMO35, ZMO44, ZMO65, ZMO85, ZMO128, ZMO135, ZMO142, ZMO145), effectively shifting all strains from strong biofilm formers to non- or weak producers.

As shown in Figure 8 and Table 10, untreated controls exhibited strong biofilm formation across all isolates, ranging from 0.543 ± 0.024 (ATCC strain) to 1.060 ± 0.010 (ZMO10 isolate), consistent with the MTP classification of strong biofilm producers. Treatment with fluconazole significantly reduced biofilm biomass ($p < 0.0001$). The ATCC strain, which is fluconazole-susceptible, decreased to 0.067 ± 0.009, placing it in the non or weak biofilm category. In contrast, the fluconazole-resistant isolates continued to form strong biofilms, with OD values ranging from 0.355 ± 0.018 (ZMO135 isolate) to 0.385 ± 0.012 (ZMO128 isolate), confirming the persistence of biofilm activity under antifungal stress.

CFSs from *Lactobacillus* strains exerted broad-spectrum inhibition across all isolates ($p < 0.0001$). *L. crispatus* reduced biofilm OD values to between 0.077 ± 0.002 (ATCC strain) and 0.250 ± 0.036 (ZMO10 isolate); *L. reuteri* suppressed biofilms from 0.075 ± 0.004 (ATCC strain) to 0.260 ± 0.037 (ZMO145 isolate); *L. delbrueckii* reduced values from 0.066 ± 0.003 (ATCC strain) to 0.251 ± 0.037 (ZMO135 isolate); and *L. fermentum* produced OD values ranging from 0.065 ± 0.003 (ATCC strain) to 0.296 ± 0.003 (ZMO10 isolate). According to MTP classification, many of these values fell into the moderate category (0.120-0.320), while several dropped below 0.120, indicating non or weak biofilm formation.

All experiments were performed in triplicate, and results are presented as mean OD values at 492 nm ± SD. Data was analysed using ANOVA followed by Tukey's multiple comparisons test to assess differences between treated groups and the untreated negative control. A p-value of < 0.05 was considered statistically significant, with all treatments demonstrating highly significant reductions in biofilm formation compared to controls ($p < 0.0001$).

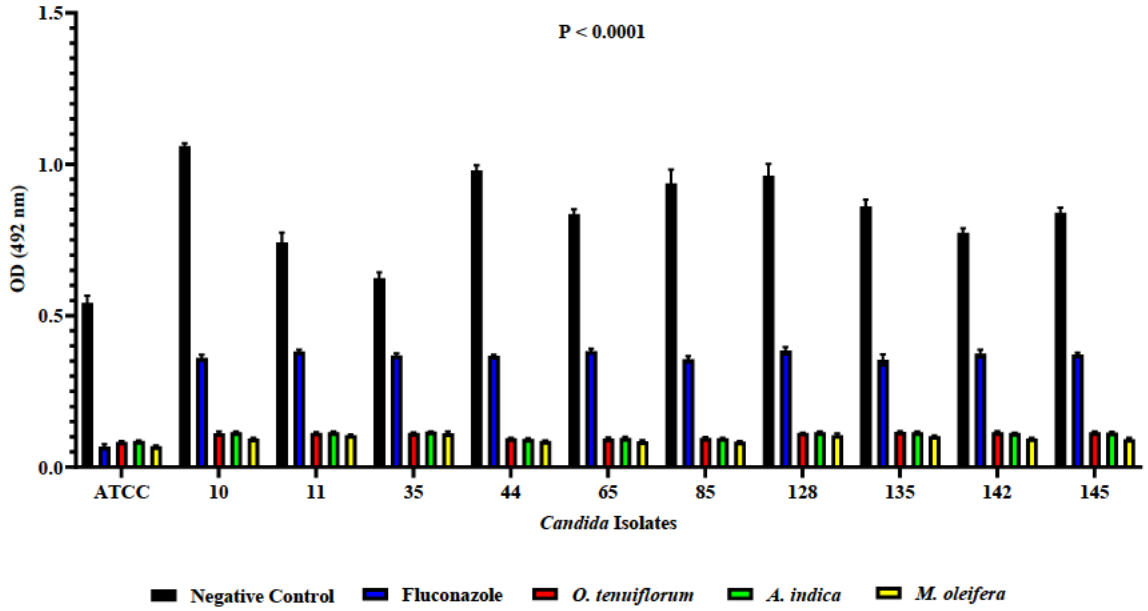

Figure 7: Biofilm formation in the fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates treated with fluconazole, *O. tenuiflorum*, *A. indica*, and *M. oleifera*. Bars represent mean OD 492 nm ± SD. Biofilm formation was determined using the MTP method, with all treatments showing significant reduction versus the negative control ($p < 0.0001$).

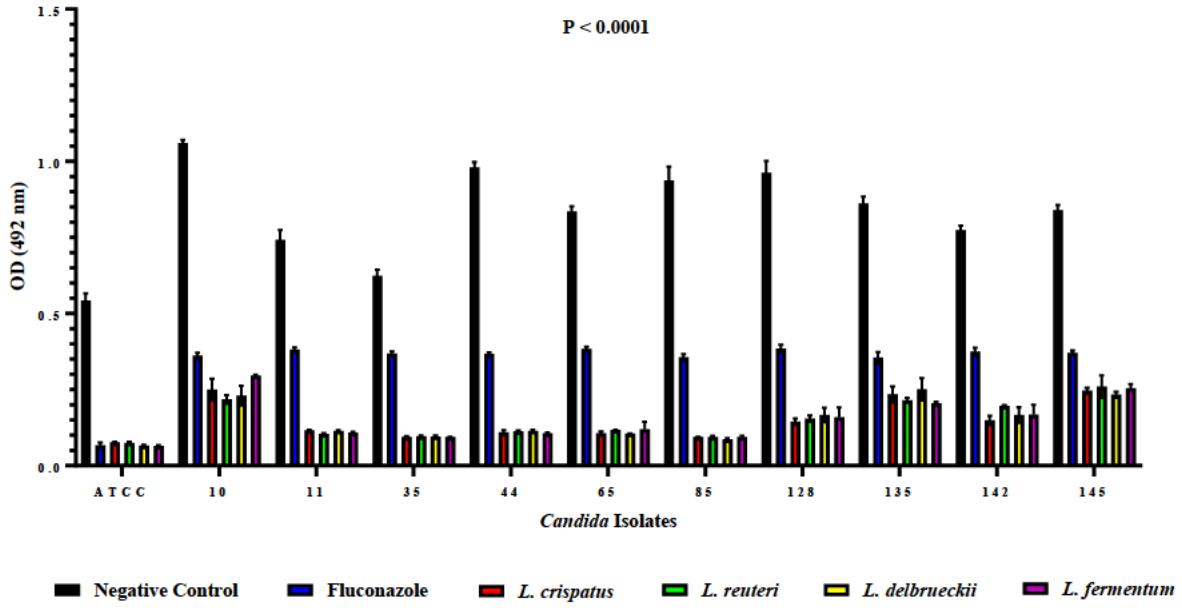

Figure 8: Biofilm formation in the fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates treated with fluconazole and CFSs from *L. crispatus*, *L. reuteri*, *L. delbrueckii*, and *L. fermentum*. Bars represent mean OD 492 nm ± SD. Biofilm formation was determined using the MTP method, with all treatments showing significant reduction versus the negative control ($p < 0.0001$).

Table 9: Mean OD values (492 nm ± SD) for the fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates under fluconazole, *O. tenuiflorum*, *A. indica*, and *M. oleifera* treatments compared with the untreated controls. Biofilm formation was classified using the MTP method, and all treatments showed significant reductions versus the negative control ($p < 0.0001$).

Isolate Name	Negative Control		Fluconazole		*O. tenuiflorum*		*A. indica*		*M. oleifera*	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
ATCC	0,543	0,024	0,067	0,009	0,083	0,002	0,086	0,003	0,069	0,002
ZMO10	1,060	0,010	0,361	0,010	0,112	0,006	0,115	0,002	0,094	0,003
ZMO11	0,741	0,033	0,381	0,007	0,113	0,002	0,115	0,003	0,105	0,003
ZMO35	0,624	0,020	0,369	0,007	0,113	0,002	0,117	0,001	0,111	0,006
ZMO44	0,980	0,018	0,367	0,004	0,095	0,002	0,093	0,003	0,086	0,003
ZMO65	0,835	0,016	0,383	0,008	0,094	0,004	0,096	0,004	0,085	0,004
ZMO85	0,937	0,046	0,356	0,011	0,096	0,003	0,095	0,002	0,084	0,002
ZMO128	0,962	0,039	0,385	0,012	0,113	0,001	0,115	0,003	0,106	0,006
ZMO135	0,861	0,023	0,355	0,018	0,117	0,002	0,115	0,003	0,102	0,003
ZMO142	0,774	0,015	0,375	0,013	0,115	0,004	0,112	0,002	0,094	0,003
ZMO145	0,840	0,017	0,371	0,007	0,115	0,003	0,114	0,003	0,092	0,004

Table 10: Mean OD values (492 nm ± SD) for the fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates under fluconazole and CFS treatments (*L. crispatus*, *L. reuteri*, *L. delbrueckii*, and *L. fermentum*) compared with untreated controls. Biofilm formation was classified using the MTP method, and all treatments showed significant reductions versus negative control ($p < 0.0001$).

Isolate Name	Negative Control		Fluconazole		*L. crispatus*		*L. reuteri*		*L. delbrueckii*		*L. fermentum*	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
ATCC	0,543	0,024	0,067	0,009	0,077	0,002	0,075	0,004	0,066	0,003	0,065	0,003
ZMO10	1,060	0,010	0,361	0,010	0,250	0,036	0,218	0,015	0,231	0,032	0,296	0,003
ZMO11	0,741	0,033	0,381	0,007	0,116	0,002	0,104	0,003	0,114	0,003	0,108	0,004
ZMO35	0,624	0,019	0,369	0,007	0,095	0,003	0,097	0,003	0,096	0,003	0,094	0,002
ZMO44	0,980	0,018	0,367	0,004	0,110	0,007	0,112	0,004	0,115	0,003	0,106	0,003
ZMO65	0,835	0,016	0,383	0,008	0,107	0,005	0,117	0,001	0,105	0,002	0,120	0,003
ZMO85	0,937	0,046	0,356	0,011	0,094	0,002	0,094	0,004	0,087	0,003	0,094	0,003
ZMO128	0,962	0,039	0,385	0,012	0,146	0,010	0,154	0,010	0,167	0,023	0,159	0,003
ZMO135	0,861	0,023	0,355	0,018	0,234	0,026	0,215	0,008	0,251	0,037	0,205	0,003
ZMO142	0,774	0,015	0,375	0,013	0,149	0,015	0,196	0,003	0,166	0,026	0,168	0,003
ZMO145	0,840	0,017	0,371	0,007	0,246	0,010	0,260	0,037	0,233	0,010	0,255	0,003

4. Discussion

Candida infections represent a major global health burden, with VVC being one of the most common mucosal fungal infections in women. It is estimated that approximately 75% of women will experience at least one episode of VVC in their lifetime, and nearly 40-50% will face at least a second episode (2, 26). More concerning is the occurrence of RVVC, defined as four or more episodes per year, which affects nearly 138 million women annually worldwide and accounts for a substantial proportion of gynaecological consultations (1). Beyond prevalence, VVC imposes significant economic and psychosocial costs, with recurrent infections leading to reduced quality of life and long-term reliance on antifungal therapy. The escalating incidence of antifungal resistance within clinical *Candida* isolates further compounds this challenge, raising urgent concerns over the sustainability of current treatment strategies (5, 27). Against this backdrop, there is a pressing need for investigations that go beyond conventional antifungal agents to identify alternative therapeutic approaches with relevance to both clinical practice and women's health globally.

This study compared conventional fluconazole with plant-derived nanoemulsions (*O. tenuiflorum*, *A. indica*, and *M. oleifera*) and CFSs from *Lactobacillus* species (*L. crispatus, L. reuteri, L. delbrueckii,* and *L. fermentum*) against biofilm formation of *C. albicans*. Using an ATCC fluconazole-susceptible strain and ten fluconazole-resistant isolates, we found that fluconazole reduced, but did not eliminate, biofilm biomass in resistant isolates. Whereas undiluted plant nanoemulsions at a concentration of 1000 μM drove OD 492 nm values below the non or weak biofilm threshold, and *Lactobacillus* CFSs inhibited growth with strain-dependent MIFs. These results reinforce two themes in contemporary mycology: azole tolerance and resistance are accentuated in the biofilm state (28), and natural plant products and *Lactobacillus* metabolites provide alternative mechanisms to disrupt *Candida* adhesion and fitness (17, 29).

Azoles remain first-line therapy for VVC, yet *C. albicans* biofilms are intrinsically more tolerant than planktonic cells (30). Mechanisms include drug sequestration in the extracellular matrix, upregulation of efflux pumps (*CDR1*, *CDR2*, *MDR1*), altered sterol biosynthesis (*ERG11*) mutations, stress responses, and chromosomal plasticity (31, 32). Our observation that resistant isolates maintained strong biofilms despite fluconazole exposure aligns with reports that azole tolerance is enhanced in biofilms compared to planktonic cells (33). Clinical reviews also emphasise that biofilm-mediated resistance is multifactorial and contributes to poor outcomes in RVVC (34). The clinical context reinforces these concerns. Centers for Disease Control and Prevention and Infectious Diseases Society of America guidelines continue to recommend azoles for uncomplicated VVC and maintenance dosing for recurrent infections (35, 36), but these treatments do not address biofilm-specific tolerance. Our finding that resistant isolates persisted as strong biofilm formers under fluconazole parallels the gap between clinical efficacy in susceptible strains and the refractory phenotype of biofilm-associated resistance.

In contrast to fluconazole, undiluted (1000 μM) nanoemulsions of *O. tenuiflorum*, *A. indica*, and *M. oleifera* consistently reduced biofilm biomass to non or weak levels across all isolates. Essential oils and phytochemicals from *Ocimum* species have been reported to inhibit *Candida* growth by disrupting membranes, inhibiting ergosterol biosynthesis, and generating oxidative stress (37, 38). *A. indica* extracts exert broad-spectrum antimicrobial effects, including anti-*Candida* activity, attributed to azadirachtin, nimbin, and related limonoids (39). *M. oleifera* contains flavonoids, alkaloids, and peptides with fungistatic and antibiofilm activity (40, 41). Our concentration-dependent outcomes agree with prior work demonstrating dose-dependent inhibition of *Candida* biofilms by plant-derived compounds (42).

Nanoemulsions likely enhance efficacy by improving the solubility, dispersion, and stability of hydrophobic phytochemicals, and by increasing the interfacial surface area, thereby facilitating contact with fungal cells. Studies have shown that nanoemulsion formulations of essential oils enhance

antifungal activity, reduce MICs, and improve antibiofilm outcomes (43, 44). Importantly, recent studies, including *Naicker et al. (2024)*, have confirmed that nanoemulsions prepared from these plants not only retain their antifungal activity but also demonstrate 0% haemolytic activity, highlighting their favourable safety profile (15). Our results mirror this delivery advantage, with strong inhibition by undiluted (1000 μM) preparations and a decline in activity with serial dilution. Although we focused on *C. albicans*, similar effects have been observed against other *Candida* species. Nanoemulsion studies highlight broad antifungal effects and potential synergy with azoles (45). Future work could explore combinatorial approaches, as synergy may allow lower dosing and reduce toxicity (46).

Beyond *O. tenuiflorum*, *A. indica*, and *M. oleifera*, other plant-based nanoemulsions have demonstrated broad-spectrum antifungal and antibiofilm activity. *Montenegro et al. (2025)* showed that a *Gomortega keule* essential oil nanoemulsion reduced MICs against both *C. albicans* and non-*albicans Candida* species, highlighting cross-species potential (45). De *Almeida et al. (2025)* reported that *Syzygium aromaticum* oil nanoemulsions co-formulated with amphotericin B achieved synergistic effects against *Candida auris* while maintaining low cytotoxicity (47). Similarly, gelatine-stabilized nanoemulsions carrying terbinafine and essential oils effectively penetrated biofilms, disrupting their structure and viability with minimal mammalian cell toxicity (48). These findings complement our results by underscoring that nanoemulsions not only improve phytochemical bioavailability but also provide versatile platforms for combination therapy. Collectively, this growing body of evidence supports nanoemulsions as promising delivery systems capable of enhancing potency, reducing host toxicity, and overcoming resistance mechanisms in *Candida* biofilms.

CFSs from *L. crispatus*, *L. reuteri*, *L. delbrueckii*, and *L. fermentum* inhibited *C. albicans* growth, though activity varied by isolate. This variation reflects known strain-dependent metabolite profiles. *Lactobacillus* species inhibits *Candida* via multiple mechanisms: lactic acid production, hydrogen peroxide, bacteriocin-like inhibitory substances, and quorum-sensing interference that blocks hyphal formation (49-51). Our finding that the fluconazole-susceptible *C. albicans* ATCC strain was generally more susceptible than the fluconazole-resistant *C. albicans* isolates agrees with reports that resistance mechanisms in clinical isolates can blunt the effects of *Lactobacillus* metabolites (17). Clinical literature supports the potential of *Lactobacillus* species in managing VVC, though results are heterogeneous. *Akinosoglou et al. (2024)* and *Liu et al. (2023)* reviewed clinical trials and concluded that *Lactobacillus* species may reduce recurrence and support antifungal therapy, but outcomes depend on strain, route, and formulation (52, 53). Our *in vitro* data provide mechanistic plausibility for the use of *Lactobacillus* species but underscore the need for carefully selected standardized strains with validated colonization capacity.

Additional studies reinforce the multifaceted mechanisms by which *Lactobacillus* metabolites inhibit *Candida* virulence. *Poon et al. (2023)* demonstrated that CFSs from *L. rhamnosus* and *L. plantarum* suppressed biofilm formation and filamentation in *C. albicans* and *C. tropicalis*, accompanied by downregulation of adhesion and morphogenesis genes (*ALS1, ALS3, EFG1,* and *TEC1*) (17). *Jang et al. (2019)* further confirmed the therapeutic potential *in vivo*, showing that CFSs from *L. crispatus* and *L. fermentum* reduced fungal burden and hyphal invasion in a murine model of VVC (54). At the molecular level, *Wang et al. (2017)* found that *L. crispatus* metabolites downregulated hyphae-associated genes (*ALS3, HWP1, ECE1*) through activation of the repressor *NRG1* (55). More recently, *MacAlpine et al. (2021)* identified 1-acetyl-beta-carboline, a small molecule secreted by *Lactobacillus*, which blocks the yeast-to-hypha transition by inhibiting the Yak1 kinase (51). Together, these findings highlight that *Lactobacillus* metabolites do not act via a single pathway, but instead modulate multiple virulence networks, supporting their potential as adjunctive therapies for recurrent and resistant VVC.

For uncomplicated VVC, azoles remain suitable first-line agents; however, recurrent and resistant cases demand alternative strategies. Our finding that plant nanoemulsions suppressed biofilm biomass across fluconazole-resistant *C. albicans* isolates indicates promise for topical formulations. Prior haemolysis studies suggest non-toxic nanoemulsions can be safely developed for mucosal use (15, 44). *Lactobacillus* species, particularly vaginal formulations containing *L. crispatus*, may complement such therapies by restoring protective microbiota (53).

To our knowledge, this is the first study to directly compare the antibiofilm effects of plant-derived nanoemulsions and *Lactobacillus* CFSs against both fluconazole-susceptible and fluconazole-resistant *C. albicans* isolates. Previous work has separately investigated plant extracts or *Lactobacillus* species against *Candida*, but none have evaluated these natural agents' side by side under the same experimental conditions. By demonstrating that fluconazole-resistant isolates maintain strong biofilms under azole treatment, while *O. tenuiflorum*, *A. indica*, and *M. oleifera* nanoemulsions completely suppressed biofilm development and *Lactobacillus* metabolites from (*L. crispatus*, *L. reuteri*, *L. delbrueckii*, and *L. fermentum*) exerted additional inhibitory activity, this study provides novel evidence of the potential for alternative strategies to overcome biofilm-associated antifungal resistance. These findings highlight an important gap in the literature and position plant-derived nanoemulsions and *Lactobacillus* metabolite-based therapies as promising adjuncts for managing VVC, particularly in the context of rising azole resistance.

5. Limitations

This study has several limitations that should be acknowledged. All experiments were conducted under *in vitro* conditions, which may not accurately reflect the complex host-microbe and immune interactions occurring *in vivo*, particularly within the vaginal environment where mucosal immunity, microbiota diversity, and pH fluctuations are influential. The study focused exclusively on *C. albicans* isolates, despite the rising clinical importance of non-*albicans Candida* species, which may exhibit different biofilm phenotypes and antifungal susceptibility profiles. Only *Lactobacillus* CFSs were tested, omitting potential synergistic effects that may arise from live *Lactobacillus* cultures through colonization, competition, or immune modulation. Lastly, the study did not assess potential synergistic or antagonistic interactions between fluconazole, plant nanoemulsions, and *Lactobacillus* metabolites, limiting insights into their combined therapeutic potential.

6. Conclusion

This study highlights the limited efficacy of fluconazole against biofilm-associated, fluconazole-resistant *C. albicans*, as resistant isolates retained strong biofilm-forming capacity even under antifungal exposure. In contrast, undiluted (1000 µM) plant-derived nanoemulsions from *O. tenuiflorum*, *A. indica*, and *M. oleifera* demonstrated potent inhibitory effects, completely suppressing biofilm development. CFSs from *Lactobacillus* strains also reduced *Candida* growth and biofilm formation, although their impact varied depending on both *Lactobacillus* strain and *Candida* isolate. Collectively, these findings support the potential of natural therapies, such as plant nanoemulsions and *Lactobacillus* supernatants, as adjuncts to conventional antifungals, particularly in cases of fluconazole-resistant VVC. Looking ahead, two key priorities emerge: the development of safe, vaginally compatible nanoemulsion formulations and the clinical translation of *Lactobacillus* strains with consistent anticandidal activity. Combination studies examining the interactions among plant nanoemulsions, *Lactobacillus* supernatants, and azole antifungals are necessary to optimise therapeutic strategies. Ultimately, *in vivo* studies and clinical trials will be critical to validate the efficacy of these natural interventions in treating biofilm-associated, recurrent, and drug-resistant VVC.

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Conflict of Interest Disclosure

The authors declare no conflict of interest.

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References

1. Denning DW, Kneale M, Sobel JD, Rautemaa-Richardson R. Global burden of recurrent vulvovaginal candidiasis: a systematic review. The Lancet infectious diseases. 2018;18(11):e339-e47.
2. Gonçalves B, Ferreira C, Alves CT, Henriques M, Azeredo J, Silva S. Vulvovaginal candidiasis: Epidemiology, microbiology and risk factors. Critical reviews in microbiology. 2016;42(6):905-27.
3. Achkar JM, Fries BC. Candida infections of the genitourinary tract. Clinical microbiology reviews. 2010;23(2):253-73.

4. Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ. Invasive candidiasis. Nature Reviews Disease Primers. 2018;4(1):1-20.
5. Perlin DS, Rautemaa-Richardson R, Alastruey-Izquierdo A. The global problem of antifungal resistance: prevalence, mechanisms, and management. The Lancet infectious diseases. 2017;17(12):e383-e92.
6. Nett JE, Andes DR. Contributions of the biofilm matrix to Candida pathogenesis. Journal of Fungi. 2020;6(1):21.
7. Arendrup MC, Patterson TF. Multidrug-resistant Candida: epidemiology, molecular mechanisms, and treatment. The Journal of infectious diseases. 2017;216(suppl_3):S445-S51.
8. Faustino C, Pinheiro L. Lipid systems for the delivery of amphotericin B in antifungal therapy. Pharmaceutics. 2020;12(1):29.
9. Rajasekar V, Darne P, Prabhune A, Kao RY, Solomon AP, Ramage G, et al. A curcumin-sophorolipid nanocomplex inhibits Candida albicans filamentation and biofilm development. Colloids and Surfaces B: Biointerfaces. 2021;200:111617.
10. Giammarino A, Verdolini L, Simonetti G, Angiolella L. Fungal Biofilm: An Overview of the Latest Nano-Strategies. Antibiotics. 2025;14(7):718.
11. Didehdar M, Chegini Z, Shariati A. Eugenol: A novel therapeutic agent for the inhibition of Candida species infection. Front Pharmacol. 2022;13:872127.
12. Mahmoud GA-E, Rashed NM, El-Ganainy SM, Salem SH. Unveiling the neem (Azadirachta indica) effects on biofilm formation of food-borne bacteria and the potential mechanism using a molecular docking approach. Plants. 2024;13(18):2669.
13. Oniha M, Eni A, Akinnola O, Omonigbehin EA, Ahuekwe EF, Olorunshola JF. In vitro antifungal activity of extracts of Moringa oleifera on phytopathogenic fungi affecting Carica papaya. Open Access Macedonian Journal of Medical Sciences. 2021;9(A):1081-5.
14. Ahmadu T, Ahmad K, Ismail S, Rashed O, Asib N, Omar D. Antifungal efficacy of Moringa oleifera leaf and seed extracts against Botrytis cinerea causing gray mold disease of tomato (Solanum lycopersicum L.). Brazilian Journal of Biology. 2020;81(4):1007-22.
15. Naicker D, Govender R, Abbai NS. Busting the Resistance: Antimicrobial Activity of Plant-Infused Nanoemulsions against Neisseria gonorrhoeae. International Journal of Microbiology. 2024;2024(1):7084347.
16. Ribeiro F, Rossoni R, De Barros P, Santos J, Fugisaki L, Leão M, et al. Action mechanisms of probiotics on Candida spp. and candidiasis prevention: an update. Journal of applied microbiology. 2020;129(2):175-85.
17. Poon Y, Hui M. Inhibitory effect of lactobacilli supernatants on biofilm and filamentation of Candida albicans, Candida tropicalis, and Candida parapsilosis. Frontiers in microbiology. 2023;14:1105949.

18. Rodríguez-Arias RJ, Guachi-Álvarez BO, Montalvo-Vivero DE, Machado A. Lactobacilli displacement and Candida albicans inhibition on initial adhesion assays: a probiotic analysis. BMC Research Notes. 2022;15(1):239.
19. Kaur DM, Patil RN, Saima A. Biosurfactant Production and Its Role in Candida albicans Biofilm Inhibition. J Pure Appl Microbiol. 2020;14(2):1337-43.
20. Ramnarain C, Sukali G, Msomi N, Mabaso N, Molatlhegi RP, Abbai NS. Correlation between genotypes and antifungal susceptibility profiles of Candida isolates from pregnant and non-pregnant women in South Africa. The Journal of Medical Laboratory Science & Technology of South Africa. 2025;7(2):6-15.
21. Hu CH, Ren LQ, Zhou Y, Ye BC. Characterization of antimicrobial activity of three Lactobacillus plantarum strains isolated from Chinese traditional dairy food. Food science & nutrition. 2019;7(6):1997-2005.
22. Rahman MM, Sazili AQ, Ahmad SA, Khalil KA, Ismail-Fitry MR, Sarker MSK. Inhibitory efficacy, production dynamics, and characterization of postbiotics of lactic acid bacteria. BMC microbiology. 2025;25(1):485.
23. Zhang X, Esmail GA, Alzeer AF, Arasu MV, Vijayaraghavan P, Choi KC, et al. Probiotic characteristics of Lactobacillus strains isolated from cheese and their antibacterial properties against gastrointestinal tract pathogens. Saudi Journal of Biological Sciences. 2020;27(12):3505-13.
24. Millsap KW, Bos R, van der Mei HC, Busscher HJ. Adhesive interactions between voice prosthetic yeast and bacteria on silicone rubber in the absence and presence of saliva. Antonie Van Leeuwenhoek. 2001;79(3-4):337-43.
25. Dhanasekaran D, Vinothini K, Latha S, Thajuddin N, Panneerselvam A. Human dental biofilm: Screening, characterization, in vitro biofilm formation and antifungal resistance of Candida spp. The Saudi Journal for Dental Research. 2014;5(1):55-70.
26. Sobel JD. Recurrent vulvovaginal candidiasis. American journal of obstetrics and gynecology. 2016;214(1):15-21.
27. Arastehfar A, Kargar ML, Mohammadi SR, Roudbary M, Ghods N, Haghighi L, et al. A high rate of recurrent vulvovaginal candidiasis and therapeutic failure of azole derivatives among Iranian women. Frontiers in microbiology. 2021;12:655069.
28. Gulati M, Nobile CJ. Candida albicans biofilms: development, regulation, and molecular mechanisms. Microbes and infection. 2016;18(5):310-21.
29. Kaur G, Chawla S, Kumar P, Singh R. Advancing vaccine strategies against Candida infections: exploring new frontiers. Vaccines. 2023;11(11):1658.
30. Gerges MA, Fahmy YA, Hosny T, Gandor NH, Mohammed SY, Mohamed TMA, et al. Biofilm Formation and Aspartyl Proteinase Activity and Their Association with Azole Resistance Among Candida albicans Causing Vulvovaginal Candidiasis, Egypt. Infection and Drug Resistance. 2023:5283-93.

31. Chaabane F, Graf A, Jequier L, Coste AT. Review on antifungal resistance mechanisms in the emerging pathogen Candida auris. Frontiers in microbiology. 2019;10:2788.
32. Lee J-H, Kim Y-G, Kim Y, Lee J. Antifungal and antibiofilm activities of chromones against nine Candida species. Microbiology Spectrum. 2023;11(6):e01737-23.
33. Sandai D, Tabana YM, El Ouweini A, Ayodeji IO. Resistance of Candida albicans biofilms to drugs and the host immune system. Jundishapur Journal of Microbiology. 2016;9(11):e37385.
34. Díaz-Navarro M, Irigoyen-von-Sierakowski Á, Delcán I, Monte A, Palomo M, Escribano P, et al. New insights in the role of Candida biofilm in the pathogenesis of recurrent vulvovaginal candidiasis: a prospective clinical study. Frontiers in Microbiology. 2025;16:1566171.
35. Workowski KA. Sexually transmitted infections treatment guidelines, 2021. MMWR Recommendations and Reports. 2021;70.
36. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, et al. Clinical practice guideline for the management of candidiasis: 2016 update by the Infectious Diseases Society of America. Clinical infectious diseases. 2016;62(4):e1-e50.
37. Khan A, Ahmad A, Akhtar F, Yousuf S, Xess I, Khan LA, et al. Ocimum sanctum essential oil and its active principles exert their antifungal activity by disrupting ergosterol biosynthesis and membrane integrity. Research in microbiology. 2010;161(10):816-23.
38. Adekunle OC, Idris OJ, Sule IO, Olorunfemi AR, Adekunle AS. Phytochemicals Combating Antibiotics Resistance: Updated Review. Alexandria Journal of Veterinary Sciences. 2022;75(1).
39. Wylie MR, Merrell DS. The Antimicrobial Potential of the Neem Tree Azadirachta indica. Front Pharmacol. 2022;13:891535.
40. Pareek A, Pant M, Gupta MM, Kashania P, Ratan Y, Jain V, et al. Moringa oleifera: An Updated Comprehensive Review of Its Pharmacological Activities, Ethnomedicinal, Phytopharmaceutical Formulation, Clinical, Phytochemical, and Toxicological Aspects. International Journal of Molecular Sciences. 2023;24(3):2098.
41. Gani BA, Soraya C, Sugiaman VK, Batubara FY, Syafriza D, Naliani S, et al. Fungistatic effect of Moringa oleifera Lam. on the metabolism changes of Candida albicans. Journal of Pharmacy & Pharmacognosy Research. 2023;11(1):179-90.
42. Bravo-Chaucanés CP, Vargas-Casanova Y, Chitiva-Chitiva LC, Ceballos-Garzon A, Modesti-Costa G, Parra-Giraldo CM. Evaluation of Anti-Candida Potential of Piper nigrum Extract in Inhibiting Growth, Yeast-Hyphal Transition, Virulent Enzymes, and Biofilm Formation. Journal of Fungi. 2022;8(8):784.
43. Krishnamoorthy R, Gassem MA, Athinarayanan J, Periyasamy VS, Prasad S, Alshatwi AA. Antifungal activity of nanoemulsion from Cleome viscosa essential oil against food-borne pathogenic Candida albicans. Saudi Journal of Biological Sciences. 2021;28(1):286-93.

44. dos Santos RD, Matos BN, Freire DO, da Silva FS, do Prado BA, Gomes KO, et al. Chemical Characterization and Antimicrobial Activity of Essential Oils and Nanoemulsions of Eugenia uniflora and Psidium guajava. Antibiotics. 2025;14(1):93.

45. Montenegro I, Fuentes B, Silva V, Valdés F, Werner E, Santander R, et al. Nanoemulsion of Gomortega keule Essential Oil: Characterization, Chemical Composition, and Anti-Yeast Activity Against Candida spp. Pharmaceutics. 2025;17(6):755.

46. Touati A, Mairi A, Ibrahim NA, Idres T. Essential Oils for Biofilm Control: Mechanisms, Synergies, and Translational Challenges in the Era of Antimicrobial Resistance. Antibiotics. 2025;14(5):503.

47. de Almeida ML, Matos APdS, Cardoso VdS, do Nascimento T, Santos-Oliveira R, Rocha LM, et al. Clove Oil-Based Nanoemulsion Containing Amphotericin B as a Therapeutic Approach to Combat Fungal Infections. Pharmaceutics. 2025;17(7):925.

48. Hassan MA, Noor S, Park J, Nabawy A, Dedhiya M, Patel R, et al. Gelatin nanoemulsion-based co-delivery of terbinafine and essential oils for treatment of Candida albicans biofilms. Microorganisms. 2025;13(1):127.

49. Takano T, Kudo H, Eguchi S, Matsumoto A, Oka K, Yamasaki Y, et al. Inhibitory effects of vaginal Lactobacilli on Candida albicans growth, hyphal formation, biofilm development, and epithelial cell adhesion. Front Cell Infect Microbiol. 2023;13:1113401.

50. Hefzy EM, Khalil MAF, Amin AAI, Ashour HM, Abdelaliem YF. Bacteriocin-Like Inhibitory Substances from Probiotics as Therapeutic Agents for Candida Vulvovaginitis. Antibiotics. 2021;10(3):306.

51. MacAlpine J, Daniel-Ivad M, Liu Z, Yano J, Revie NM, Todd RT, et al. A small molecule produced by Lactobacillus species blocks Candida albicans filamentation by inhibiting a DYRK1-family kinase. Nature Communications. 2021;12(1):6151.

52. Akinosoglou K, Schinas G, Polyzou E, Tsiakalos A, Donders GGG. Probiotics in the Management of Vulvovaginal Candidosis. Journal of Clinical Medicine. 2024;13(17):5163.

53. Liu Z, Yang H, Huang R, Li X, Sun T, Zhu L. Vaginal mycobiome characteristics and therapeutic strategies in vulvovaginal candidiasis (VVC): differentiating pathogenic species and microecological features for stratified treatment. Clinical Microbiology Reviews. 2025;38(2):e00284-24.

54. Jang SJ, Lee K, Kwon B, You HJ, Ko G. Vaginal lactobacilli inhibit growth and hyphae formation of Candida albicans. Sci Rep. 2019;9(1):8121.

55. Wang S, Wang Q, Yang E, Yan L, Li T, Zhuang H. Antimicrobial Compounds Produced by Vaginal Lactobacillus crispatus Are Able to Strongly Inhibit Candida albicans Growth, Hyphal Formation and Regulate Virulence-related Gene Expressions. Front Microbiol. 2017;8:564.

Supplementary Material

Supplementary Table 1: Growth response of fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates to the *O. tenuiflorum* nanoemulsion at different concentrations (1000-1 µM).

O. tenuiflorum				
Isolate Name	**1000 µM**	**100 µM**	**10 µM**	**1 µM**
ATCC	No Growth	Growth	Growth	Growth
ZMO10	No Growth	Growth	Growth	Growth
ZMO11	No Growth	Growth	Growth	Growth
ZMO35	No Growth	Growth	Growth	Growth
ZMO44	No Growth	Growth	Growth	Growth
ZMO65	No Growth	Growth	Growth	Growth
ZMO85	No Growth	Growth	Growth	Growth
ZMO128	No Growth	Growth	Growth	Growth
ZMO135	No Growth	Growth	Growth	Growth
ZMO142	No Growth	Growth	Growth	Growth
ZMO145	No Growth	Growth	Growth	Growth

Supplementary Table 2: Growth response of the fluconazole-susceptible *C. albicans* ATCC strain and the ten fluconazole-resistant *C. albicans* isolates to the *A. indica* nanoemulsion at different concentrations (1000-1 µM).

A. indica				
Isolate Name	**1000 µM**	**100 Mm**	**10 µM**	**1 µM**
ATCC	No Growth	Growth	Growth	Growth
ZMO10	No Growth	Growth	Growth	Growth
ZMO11	No Growth	Growth	Growth	Growth
ZMO35	No Growth	Growth	Growth	Growth
ZMO44	No Growth	Growth	Growth	Growth
ZMO65	No Growth	Growth	Growth	Growth
ZMO85	No Growth	Growth	Growth	Growth
ZMO128	No Growth	Growth	Growth	Growth
ZMO135	No Growth	Growth	Growth	Growth
ZMO142	No Growth	Growth	Growth	Growth
ZMO145	No Growth	Growth	Growth	Growth

Supplementary Table 3: Growth response of the fluconazole-susceptible *C. albicans* ATCC strain and the ten *C. albicans* fluconazole-resistant isolates to the *M. oleifera* nanoemulsion at different concentrations (1000-1 µM).

M. oleifera				
Isolate Name	**1000 µM**	**100 µM**	**10 µM**	**1 µM**
ATCC	No Growth	Growth	Growth	Growth
ZMO10	No Growth	Growth	Growth	Growth
ZMO11	No Growth	Growth	Growth	Growth
ZMO35	No Growth	Growth	Growth	Growth
ZMO44	No Growth	Growth	Growth	Growth
ZMO65	No Growth	Growth	Growth	Growth
ZMO85	No Growth	Growth	Growth	Growth
ZMO128	No Growth	Growth	Growth	Growth
ZMO135	No Growth	Growth	Growth	Growth
ZMO142	No Growth	Growth	Growth	Growth
ZMO145	No Growth	Growth	Growth	Growth

CHAPTER 5

Synthesis

Understanding the epidemiological, phenotypic, genotypic, and molecular characteristics of *C. albicans* remains essential for addressing the growing global challenge of VVC. The increasing burden of antifungal resistance, coupled with expanding treatment failures, recurrent disease patterns, and changing host demographics, has transformed VVC from a typically manageable superficial infection into one with significant public health implications (Sobel, 2016; Denning *et al.*, 2018). Globally, millions of women experience RVVC annually, with fluconazole resistance emerging as a major contributor to therapeutic failure (de Cássia Orlandi Sardi *et al.*, 2021). In South Africa, the challenge is compounded by high rates of HIV infection, altered immunity, and restricted access to diverse antifungal agents, placing women at particularly high risk for symptomatic and recurrent disease (Naicker *et al.*, 2016; Barnabas, 2019). Despite this burden, local data characterising *C. albicans* strain diversity, antifungal susceptibility patterns, biofilm-forming capacity, and molecular resistance mechanisms remain limited. This knowledge gap hinders the development of context-specific diagnostic and therapeutic guidelines. The present thesis therefore sought to provide a comprehensive, multidimensional assessment of *C. albicans* isolates from South African women using integrated genotypic, phenotypic, molecular, and therapeutic approaches to generate a holistic understanding of strain behaviour, resistance mechanisms and potential alternative treatments.

Central to the persistence and pathogenicity of *C. albicans* is its remarkable genetic plasticity, which allows it to rapidly adapt under selective pressures such as host immunity, environmental stress, and antifungal exposure (Nobile and Johnson, 2015). This organism possesses a highly flexible genome capable of undergoing microevolutionary shifts, phenotypic switching, chromosomal rearrangements, and biofilm formation, all of which contribute to its dual ability to exist as both a commensal and a pathogen (Mayer, Duncan and and Hube, 2013). As azole antifungals remain the first-line therapy in many regions, including South Africa, widespread and often repeated fluconazole exposure has intensified selective pressure, accelerating the emergence of resistance (Whaley, 2018). Monitoring resistance mechanisms within local *Candida* populations is thus urgently required to ensure accurate surveillance, inform clinical decision-making, and maintain effective treatment strategies.

Globally, the increase in fluconazole resistance among *C. albicans* isolates is driven by several well-characterised mechanisms. These include the upregulation of efflux pumps, mutations and overexpression of the *ERG11* gene, alterations in ergosterol biosynthesis, and an enhanced capacity for biofilm formation (Perlin, Rautemaa-Richardson and Alastruey-Izquierdo, 2017; Bhattacharya, Sae-Tia and Fries, 2020). As each mechanism contributes uniquely to resistance severity and treatment outcome, it is necessary to examine strain behaviour across multiple analytical levels, epidemiological, phenotypic, molecular, and therapeutic, to gain a comprehensive understanding of local resistance

dynamics. While international literature has extensively explored these mechanisms, very few studies have contextualised them in South African women. This reality formed a significant motivation for the present research project.

A foundational step in understanding *C. albicans* behaviour involves characterising strain diversity. ABC genotyping, which classifies isolates based on size polymorphism within the *25S rDNA* region, remains a globally recognised tool used to explore strain variation and potential links to virulence, epidemiology, and antifungal resistance (McCullough, Clemons and Stevens, 1999; Fornari *et al.*, 2016). Several international studies suggest that genotypes A, B, and C may differ in pathogenic potential, capacity for antifungal resistance, or association with recurrent infections (Gerós-Mesquita *et al.*, 2020; Gharaghani *et al.*, 2022). However, in South Africa, genotyping is not routinely incorporated into *Candida* surveillance, contributing to limited knowledge regarding local strain distributions and their potential clinical implications. The present study, therefore, undertook a detailed analysis of its genotypic patterns to expand the understanding of local *C. albicans* population structure.

The genotyping results revealed that genotype A was the most common genotype circulating in this cohort, a finding consistent with global and African studies (da Silva-Rocha *et al.*, 2014; Ali-Shtayeh *et al.*, 2015; Fornari *et al.*, 2016). Genotype A strains have been characterised as highly adaptable, often exhibiting strong biofilm formation, enhanced virulence, and greater tolerance to antifungal stress (Zhang *et al.*, 2020; Al-Groom, Ali and Shaqra, 2024). These traits may provide genotype A strains with ecological advantages within the vaginal niche, particularly among women with disrupted microbiota, hormonal fluctuations, or immune compromise (Gerós-Mesquita *et al.*, 2020; Gharaghani *et al.*, 2022). The relatively lower prevalence of genotypes B and C is also consistent with global patterns, where these groups appear less frequently in both commensal and clinical isolates (Muadcheingka and Tantivitayakul, 2015; Ali, Ghamry and Ibrahim, 2024). The presence of all three genotypes, however, highlights underlying genetic heterogeneity within the population and underscores the importance of continued molecular surveillance to detect shifting trends, especially as antifungal resistance increases.

A notable observation in this study was the presence of fluconazole-resistant isolates predominantly within genotype A. Although ABC genotyping cannot independently predict antifungal susceptibility, several studies have reported trends linking genotype A with reduced susceptibility to azoles (Fornari *et al.*, 2016; Al-Groom, Ali and Shaqra, 2024). The evolutionary versatility of genotype A may facilitate the acquisition or induction of resistance-associated mechanisms, particularly under sustained azole pressure (Whaley, 2018; Fisher *et al.*, 2022). This pattern highlights the multifaceted nature of fluconazole resistance, influenced by genetic composition, environmental exposure, and clinical practices (Perlin, Rautemaa-Richardson and Alastruey-Izquierdo, 2017; Pfaller *et al.*, 2019). The genotyping findings generated in this thesis, therefore, provide valuable insight into local resistance evolution and reinforce the need for genotype-informed surveillance.

While genotyping provides critical epidemiological insights, phenotypic susceptibility testing remains essential for evaluating the potential clinical response. The fluconazole minimum inhibitory concentration (MIC) patterns observed in this study demonstrated the presence of reduced susceptibility and resistance, consistent with reports from various African regions (Africa and Abrantes, 2016; Mushi *et al.*, 2019). As RVVC is strongly associated with azole exposure, rising resistance is particularly concerning. Fluconazole, while widely used, is known to have limited activity against biofilm-associated cells, and resistance in planktonic populations often predicts even poorer outcomes in established infections (Wall *et al.*, 2019). The phenotypic resistance patterns identified in this thesis, therefore, highlight an emerging challenge within South African clinical settings, especially in areas where antifungal options remain limited.

To complement the phenotypic findings, molecular evaluation of the *ERG11* gene expression provided deeper insight into the mechanisms underpinning resistance. Upregulation of *ERG11* is a well-documented azole resistance mechanism that reduces fluconazole binding efficiency and facilitates continued ergosterol production despite drug exposure (Flowers *et al.*, 2015; Nishimoto, Sharma and Rogers, 2019). The consistent overexpression of *ERG11* observed among resistant isolates in this study reinforces the hypothesis that transcriptional upregulation, rather than point mutations alone, may be a dominant resistance mechanism within the local population (Whaley, 2018; Lee *et al.*, 2020). This aligns with global research suggesting that increased *ERG11* transcription is particularly prevalent in settings where fluconazole is widely used as the primary antifungal agent (Arastehfar *et al.*, 2020; Doorley *et al.*, 2023). The molecular findings in this thesis, therefore, contribute crucial new knowledge to the limited pool of South African resistance research, supporting the integration of gene expression profiling into future diagnostic and surveillance frameworks.

Phylogenetic analysis provided additional insight into the evolutionary dynamics of resistance. The clustering of resistant isolates into distinct sub-groups suggests that resistance may not emerge randomly but instead may be concentrated within specific evolutionary lineages (Tian *et al.*, 2021; Gong *et al.*, 2023). These findings mirror international studies demonstrating that resistant strains often cluster together, indicating either shared ancestry or convergent evolution under antifungal pressure (Muñoz *et al.*, 2021). The alignment of elevated *ERG11* expression with phylogenetic clustering strengthens the argument that both functional and evolutionary drivers contribute to resistance patterns in this population. This reinforces the broader clinical concern that resistant strains may persist within communities, particularly in settings where empirical fluconazole therapy is standard.

The rise of antifungal resistance further underscores the need to explore novel therapeutic options that can address the limitations of conventional azoles. The natural therapeutics component of this study, therefore, adds significant translational value to the broader research trajectory. Plant-derived nanoemulsions have gained increased interest due to their ability to enhance the solubility, stability, and

bioavailability of phytochemicals with known antifungal properties (Gao *et al.*, 2024; Zafar *et al.*, 2024). In this study, plant nanoemulsions formulated from *O. tenuiflorum*, *A. indica* and *M. oleifera* demonstrated noteworthy inhibitory activity against planktonic *C. albicans*, exhibiting particularly strong antifungal effects. These findings align closely with international literature, which reports the potent antimicrobial activity of *A. indica*-derived compounds, such as nimbidin and azadirachtin, as well as nanoemulsions derived from *O. tenuiflorum* and *M. oleifera* (Khan and Javed, 2021; Naicker, Govender and Abbai, 2024).

Equally important was the nanoemulsions' ability to disrupt preformed biofilms. Biofilms remain one of the most significant challenges in VVC management, contributing significantly to resistant infections, recurrent disease, and treatment failure (Nett and Andes, 2020; Lohse *et al.*, 2020). The enhanced penetration and delivery capacity of nanoemulsions likely contributed to their improved antibiofilm effects, supporting the concept that nanotechnology can overcome some of the protective barriers associated with fungal biofilms (Rao *et al.*, 2021). These findings provide a compelling argument for the development of topical nanoemulsion-based formulations as adjunctive therapies for VVC, especially in cases where azole resistance undermines treatment efficacy.

Parallel to the plant-based formulations, the *Lactobacillus* CFSs evaluated in this study demonstrated strong antifungal and antibiofilm activity. *Lactobacilli* play a central role in maintaining vaginal homeostasis through the production of lactic acid, bacteriocins, and hydrogen peroxide, which inhibit pathogenic microbes (van de Wijgert and Verwijs, 2020; Poon and Hui, 2023). The inhibition of growth and early biofilm formation observed in this study supports growing international evidence that probiotic-derived metabolites can inhibit azole-resistant strains (Ribeiro *et al.*, 2020; Parolin *et al.*, 2022). These findings demonstrate the feasibility of microbiota-based therapeutics as low-cost, biologically compatible options for managing RVVC or resistant VVC.

Taken together, the integrated findings of this thesis reveal a complex and evolving landscape in which *C. albicans* infections are driven by interactions between strain diversity, resistance mechanisms, antifungal exposure, and biofilm-associated survival. The predominance of genotype A, the detection of resistant isolates, the confirmed overexpression of *ERG11*, and the demonstrated antibiofilm potential of natural therapeutics collectively provide a strong scientific foundation for advancing diagnostic and therapeutic strategies within South Africa. The convergence of these elements underscores the need for updated treatment guidelines that incorporate susceptibility testing, molecular surveillance, and integrative therapeutic approaches. In settings where fluconazole remains the only widely accessible antifungal, exploring alternative and adjunctive therapies becomes essential to mitigate the spread of resistance, reduce recurrence, and improve treatment outcomes.

Overall, this thesis presents a multidimensional, contextually relevant, and scientifically robust assessment of *C. albicans* in South African women. By combining genotypic, phenotypic, molecular

and therapeutic insights, it advances the understanding of resistance evolution and provides promising new avenues for effective management of VVC in resource-limited settings.

Strengths of the study

This thesis possesses several notable strengths that enhance its scientific value, methodological rigour, and contribution to the field of medical microbiology. One of the most significant strengths is the comprehensive and multi-dimensional approach undertaken to characterise *C. albicans* isolates from South African women. By integrating phenotypic antifungal susceptibility testing, ABC genotyping, quantitative gene expression analysis, and therapeutic evaluation using both plant-based nanoemulsions and *Lactobacillus* CFSs, this research provides a holistic and layered understanding of strain behaviour, resistance mechanisms, and treatment vulnerabilities. Very few local studies have combined these methodologies within a single project, making this thesis one of the most extensive evaluations of *C. albicans* in a South African clinical population.

Another key strength lies in the use of locally collected clinical isolates, which ensures that the findings are directly relevant to South African women and reflective of the unique epidemiological landscape shaped by high HIV prevalence, RVVC burden, and widespread use of azole antifungals. The inclusion of both pregnant and non-pregnant women further strengthens the generalisability of the findings. Additionally, the molecular component of the study provides novel local evidence of *ERG11* overexpression and phylogenetic clustering, thereby enhancing understanding of resistance mechanisms in this region.

Methodologically, the study benefits from rigorous laboratory procedures, appropriate controls, and adherence to recognised Clinical and Laboratory Standards Institute (CLSI) guidelines for antifungal susceptibility testing. The incorporation of nanoemulsion technology and probiotic-derived metabolites represents an innovative therapeutic exploration that adds translational value. Together, these strengths position the thesis as a robust and impactful contribution to the literature on *C. albicans* epidemiology, resistance, and alternative therapeutics.

Limitations of the study

Despite its strengths, this study has limitations. One limitation relates to the restricted number of fluconazole-resistant isolates included in the gene expression and phylogenetic analyses. Although these isolates were selected to represent the observed resistance patterns, the small sample size, particularly the limited number of resistant isolates evaluated, may have reduced statistical power and constrained the identification of additional molecular or evolutionary trends. Similarly, the study did not investigate efflux pump activity, point mutations within *ERG11* or other genes implicated in azole resistance (e.g., *CDR1*, *CDR2*, *MDR1*), which may complement the expression findings and further clarify resistance pathways.

Another limitation is that the nanoemulsion and CFS assays were conducted under *in vitro* conditions, which, while informative, may not fully capture the complexity of the vaginal microenvironment. Factors such as mucosal immunity, microbial interactions, and host hormonal influences were not assessed but may influence therapeutic effectiveness *in vivo*. Additionally, the study did not evaluate potential synergistic effects between natural therapeutics and fluconazole, which could provide clinically relevant insights into combination therapy strategies.

Finally, this research was conducted at a single clinical site in KwaZulu-Natal, which may limit the generalisation of findings to other regions with different demographic or clinical characteristics. Nonetheless, these limitations do not diminish the value of the study; instead, they highlight important areas for future research aimed at expanding the understanding of *C. albicans* epidemiology and therapy within South Africa.

Conclusion

This thesis provides a comprehensive and integrated understanding of *C. albicans* infections among South African women by examining the organism from a multifaceted approach that incorporates genotypic diversity, phenotypic susceptibility, molecular resistance mechanisms, and the potential of novel therapeutic strategies. Together, these findings create a unified scientific narrative that reflects both the complexity and the clinical urgency of VVC in settings where antifungal resistance is rising and treatment options remain limited.

The study revealed that *C. albicans* isolates circulating within this population exhibit notable genetic diversity, with genotype A emerging as the predominant lineage. This genotype not only reflected global distribution patterns but was also observed among isolates demonstrating reduced susceptibility to fluconazole, suggesting a possible link between genotype and resistance potential. These findings highlight the importance of monitoring strain distribution and its relationship to antifungal response, particularly in regions where azole exposure is widespread.

Phenotypic susceptibility testing further demonstrated that some isolates exhibited elevated fluconazole MIC values, reinforcing growing international concerns regarding reduced azole susceptibility in *C. albicans*. This trend was more clearly understood through molecular analyses, which showed significant upregulation of *ERG11* in resistant isolates. The overexpression of *ERG11*, a well-established mechanism of azole resistance, confirmed that resistance in this cohort is at least partly driven by transcriptional adaptation, reflecting the selective pressure of prolonged azole use. Phylogenetic clustering of resistant isolates also suggested that resistance traits may be conserved within specific evolutionary lineages, pointing to a need for ongoing molecular surveillance.

Beyond characterising resistance, this thesis explored therapeutic alternatives capable of addressing the challenges posed by fluconazole resistance and biofilm-associated tolerance. The plant-based

nanoemulsions formulated from *O. tenuiflorum*, *A. indica*, and *M. oleifera* demonstrated encouraging antifungal and antibiofilm activity. These findings are significant considering the persistence of biofilm-associated infections and the limited efficacy of conventional antifungals against biofilm-embedded cells. Complementing this, the *Lactobacillus* CFSs exhibited substantial inhibitory activity against both planktonic growth and early biofilm formation, aligning with global evidence supporting the protective role of probiotic metabolites in vaginal health.

Taken together, the collective findings of this thesis highlight the multifaceted nature of VVC and reinforce the need for integrated approaches that combine molecular surveillance, improved diagnostic accuracy, and the development of new therapeutic modalities. By generating locally relevant evidence and evaluating practical, low-cost treatment alternatives, this work makes a meaningful contribution to addressing the emerging threat of antifungal resistance within South African clinical and public health settings. Ultimately, the thesis highlights the importance of ongoing interdisciplinary research to support effective clinical management and inform future therapeutic innovations for women affected by recurrent or drug-resistant *C. albicans* infections.

Future recommendations

Future research should prioritise expanded molecular surveillance to track the evolution and dissemination of resistant *C. albicans* lineages, including larger-scale genomic studies to identify additional resistance determinants beyond *ERG11* overexpression. Routine antifungal susceptibility testing should be incorporated into clinical practice to guide evidence-based treatment decisions, particularly in settings where fluconazole is widely used as first-line therapy. Further exploration of plant-based nanoemulsions and *Lactobacillus* metabolites is warranted, including optimisation of formulations, *in vivo* validation, and the development of clinical-grade therapeutic prototypes suitable for vaginal application. Integrating these alternative therapeutics into combination strategies alongside conventional antifungals may help overcome biofilm-associated resistance and reduce recurrence rates. Additionally, greater attention should be directed toward understanding host-microbiome interactions and immunological factors influencing susceptibility to VVC, as these insights could inform personalised interventions aimed at restoring vaginal homeostasis. Together, these recommendations pave the way for developing accessible, effective, and sustainable solutions to address the rising burden of antifungal-resistant *C. albicans* in South Africa and beyond.

REFERENCES

Africa, C. W. and Abrantes, P. M. (2016) 'Candida antifungal drug resistance in sub-Saharan African populations: A systematic review', *F1000Res,* 5, pp. 2832.

Al-Groom, R. M., Ali, R. R. M. and Shaqra, Q. M. A. (2024) 'Genotypes analysis and antifungal susceptibility of Candida albicans strains isolated from women with vaginal candidiasis in Jordan using PCR targeting 25SrDNA and ALT repeat sequences of the RPS', *Pakistan Journal of Medical Sciences,* 40(8), pp. 1619.

Ali-Shtayeh, M., Jamous, R., Alothman, N., Baker, M., Zaitoun, S., Mallah, O. and Jamous, R. (2015) 'Genotyping and antifungal susceptibility of Candida albicans strains from patients with vulvovaginal and cutaneous candidiasis in Palestine'.

Ali, S. A.-S. I., Ghamry, A. A. and Ibrahim, F. A. (2024) 'ABC genotyping, virulence factors production and antifungal resistant pattern of Candida albicans isolated from immunocompromised patients with candidemia', *Microbes and Infectious Diseases,* 5(4), pp. 1621–1630.

Arastehfar, A., Gabaldón, T., Garcia-Rubio, R., Jenks, J. D., Hoenigl, M., Salzer, H. J. F., Ilkit, M., Lass-Flörl, C. and Perlin, D. S. (2020) 'Drug-Resistant Fungi: An Emerging Challenge Threatening Our Limited Antifungal Armamentarium', *Antibiotics,* 9(12), pp. 877.

Barnabas, S. L. (2019) 'Sexually transmitted infections, bacterial vaginosis and genital inflammation as risk factors of HIV acquisition in adolescent girls and young women in South Africa'.

Bhattacharya, S., Sae-Tia, S. and Fries, B. C. (2020) 'Candidiasis and Mechanisms of Antifungal Resistance', *Antibiotics (Basel),* 9(6).

da Silva-Rocha, W. P., Lemos, V. L. d. B., Svidizisnki, T. I. E., Milan, E. P. and Chaves, G. M. (2014) 'Candida species distribution, genotyping and virulence factors of Candida albicans isolated from the oral cavity of kidney transplant recipients of two geographic regions of Brazil', *BMC Oral Health,* 14(1), pp. 20.

de Cássia Orlandi Sardi, J., Silva, D. R., Anibal, P. C., de Campos Baldin, J. J. C. M., Ramalho, S. R., Rosalen, P. L., Macedo, M. L. R. and Hofling, J. F. (2021) 'Vulvovaginal candidiasis: epidemiology and risk factors, pathogenesis, resistance, and new therapeutic options', *Current Fungal Infection Reports,* 15(1), pp. 32–40.

Denning, D. W., Kneale, M., Sobel, J. D. and Rautemaa-Richardson, R. (2018) 'Global burden of recurrent vulvovaginal candidiasis: a systematic review', *The Lancet infectious diseases,* 18(11), pp. e339–e347.

Doorley, L. A., Barker, K. S., Zhang, Q., Rybak, J. M. and Rogers, P. D. (2023) 'Mutations in TAC1 and ERG11 are major drivers of triazole antifungal resistance in clinical isolates of Candida parapsilosis', *Clinical Microbiology and Infection,* 29(12), pp. 1602. e1–1602. e7.

Fisher, M. C., Alastruey-Izquierdo, A., Berman, J., Bicanic, T., Bignell, E. M., Bowyer, P., Bromley, M., Brüggemann, R., Garber, G. and Cornely, O. A. (2022) 'Tackling the emerging threat of antifungal resistance to human health', *Nature reviews microbiology,* 20(9), pp. 557–571.

Flowers, S. A., Colón, B., Whaley, S. G., Schuler, M. A. and Rogers, P. D. (2015) 'Contribution of clinically derived mutations in ERG11 to azole resistance in Candida albicans', *Antimicrob Agents Chemother,* 59(1), pp. 450–60.

Fornari, G., Vicente, V. A., Gomes, R. R., Muro, M. D., Pinheiro, R. L., Ferrari, C., Herkert, P. F., Takimura, M., Carvalho, N. S. d. and Queiroz-Telles, F. (2016) 'Susceptibility and molecular characterization of Candida species from patients with vulvovaginitis', *brazilian journal of microbiology,* 47, pp. 373–380.

Gao, Y., Cao, Q., Xiao, Y., Wu, Y., Ding, L., Huang, H., Li, Y., Yang, J. and Meng, L. (2024) 'The progress and future of the treatment of Candida albicans infections based on nanotechnology', *Journal of Nanobiotechnology,* 22(1), pp. 568.

Gerós-Mesquita, Â., Carvalho-Pereira, J., Franco-Duarte, R., Alves, A., Gerós, H., Pais, C. and Sampaio, P. (2020) 'Oral Candida albicans colonization in healthy individuals: prevalence, genotypic diversity, stability along time and transmissibility', *Journal of Oral Microbiology,* 12(1), pp. 1820292.

Gharaghani, M., Shabanzadeh, M., Jafarian, H. and Zarei Mahmoudabadi, A. (2022) 'ABC typing and extracellular enzyme production of Candida albicans isolated from Candida vulvovaginitis', *Journal of Clinical Laboratory Analysis,* 36(1), pp. e24117.

Gong, J., Chen, X.-F., Fan, X., Xu, J., Zhang, H., Li, R.-Y., Chen, S. C.-A., Kong, F., Zhang, S., Sun, Z.-Y., Kang, M., Liao, K., Guo, D.-W., Wan, Z., Hu, Z.-D., Chu, Y.-Z., Zhao, H.-M., Zou, G.-L., Shen, C., Geng, Y.-Y., Wu, W.-W., Wang, H., Zhao, F., Lu, X., He, L.-H., Liu, G.-M., Xu, Y.-C., Zhang, J.-Z. and Xiao, M. (2023) 'Emergence of Antifungal Resistant Subclades in the Global Predominant Phylogenetic Population of Candida albicans', *Microbiology Spectrum,* 11(1), pp. e03807–22.

Khan, K. and Javed, S. (2021) 'Silver nanoparticles synthesized using leaf extract of Azadirachta indica exhibit enhanced antimicrobial efficacy than the chemically synthesized nanoparticles: A comparative study', *Science Progress,* 104(2), pp. 00368504211012159.

Lee, Y., Puumala, E., Robbins, N. and Cowen, L. E. (2020) 'Antifungal drug resistance: molecular mechanisms in Candida albicans and beyond', *Chemical Reviews,* 121(6), pp. 3390–3411.

Lohse, M. B., Gulati, M., Craik, C. S., Johnson, A. D. and Nobile, C. J. (2020) 'Combination of antifungal drugs and protease inhibitors prevent Candida albicans biofilm formation and disrupt mature biofilms', *Frontiers in Microbiology,* 11, pp. 1027.

Mayer, F. L., Duncan, W. and and Hube, B. (2013) 'Candida albicans pathogenicity mechanisms', *Virulence,* 4(2), pp. 119–128.

McCullough, M. J., Clemons, K. V. and Stevens, D. A. (1999) 'Molecular and phenotypic characterization of genotypic Candida albicans subgroups and comparison with Candida dubliniensis and Candida stellatoidea', *Journal of clinical microbiology,* 37(2), pp. 417–421.

Muadcheingka, T. and Tantivitayakul, P. (2015) 'Distribution of Candida albicans and non-albicans Candida species in oral candidiasis patients: Correlation between cell surface hydrophobicity and biofilm forming activities', *Archives of oral biology,* 60(6), pp. 894–901.

Muñoz, J. F., Welsh, R. M., Shea, T., Batra, D., Gade, L., Howard, D., Rowe, L. A., Meis, J. F., Litvintseva, A. P. and Cuomo, C. A. (2021) 'Clade-specific chromosomal rearrangements and loss of subtelomeric adhesins in Candida auris', *Genetics,* 218(1).

Mushi, M. F., Bader, O., Bii, C., Groß, U. and Mshana, S. E. (2019) 'Virulence and susceptibility patterns of clinical Candida spp. isolates from a tertiary hospital, Tanzania', *Medical mycology,* 57(5), pp. 566–572.

Naicker, D., Govender, R. and Abbai, N. S. (2024) 'Busting the Resistance: Antimicrobial Activity of Plant-Infused Nanoemulsions against Neisseria gonorrhoeae', *International Journal of Microbiology,* 2024(1), pp. 7084347.

Naicker, S. D., Govender, N., Patel, J., Zietsman, I. L., Wadula, J., Coovadia, Y., Kularatne, R., Seetharam, S., Govender, N. P. and group, T.-S. (2016) 'Comparison of species-level identification and antifungal susceptibility results from diagnostic and reference laboratories for bloodstream Candida surveillance isolates, South Africa, 2009-2010', *Sabouraudia,* 54(8), pp. 816–824.

Nett, J. E. and Andes, D. R. (2020) 'Contributions of the biofilm matrix to Candida pathogenesis', *Journal of Fungi,* 6(1), pp. 21.

Nishimoto, A. T., Sharma, C. and Rogers, P. D. (2019) 'Molecular and genetic basis of azole antifungal resistance in the opportunistic pathogenic fungus Candida albicans', *Journal of Antimicrobial Chemotherapy,* 75(2), pp. 257–270.

Nobile, C. J. and Johnson, A. D. (2015) 'Candida albicans biofilms and human disease', *Annual review of microbiology,* 69(1), pp. 71–92.

Parolin, C., Croatti, V., Giordani, B. and Vitali, B. (2022) 'Vaginal Lactobacillus Impair Candida Dimorphic Switching and Biofilm Formation', *Microorganisms,* 10(10), pp. 2091.

Perlin, D. S., Rautemaa-Richardson, R. and Alastruey-Izquierdo, A. (2017) 'The global problem of antifungal resistance: prevalence, mechanisms, and management', *The Lancet infectious diseases,* 17(12), pp. e383–e392.

Pfaller, M. A., Diekema, D. J., Turnidge, J. D., Castanheira, M. and Jones, R. N. (2019) 'Twenty Years of the SENTRY Antifungal Surveillance Program: Results for Candida Species From 1997–2016', *Open Forum Infectious Diseases,* 6(Supplement_1), pp. S79–S94.

Poon, Y. and Hui, M. (2023) 'Inhibitory effect of lactobacilli supernatants on biofilm and filamentation of Candida albicans, Candida tropicalis, and Candida parapsilosis', *Frontiers in Microbiology,* 14, pp. 1105949.

Rao, H., Choo, S., Rajeswari Mahalingam, S. R., Adisuri, D. S., Madhavan, P., Md. Akim, A. and Chong, P. P. (2021) 'Approaches for Mitigating Microbial Biofilm-Related Drug Resistance: A Focus on Micro- and Nanotechnologies', *Molecules,* 26(7), pp. 1870.

Ribeiro, F., Rossoni, R., De Barros, P., Santos, J., Fugisaki, L., Leão, M. and Junqueira, J. (2020) 'Action mechanisms of probiotics on Candida spp. and candidiasis prevention: an update', *Journal of Applied Microbiology,* 129(2), pp. 175–185.

Sobel, J. D. (2016) 'Recurrent vulvovaginal candidiasis', *American Journal of Obstetrics and Gynecology,* 214(1), pp. 15–21.

Tian, J.-y., Yang, Y.-g., Chen, S., Teng, Y. and Li, X.-z. (2021) 'Genetic diversity and molecular epidemiology of Candida albicans from vulvovaginal candidiasis patients', *Infection, Genetics and Evolution,* 92, pp. 104893.

van de Wijgert, J. H. and Verwijs, M. C. (2020) 'Lactobacilli-containing vaginal probiotics to cure or prevent bacterial or fungal vaginal dysbiosis: a systematic review and recommendations for future trial designs', *BJOG: An International Journal of Obstetrics & Gynaecology,* 127(2), pp. 287–299.

Wall, G., Montelongo-Jauregui, D., Bonifacio, B. V., Lopez-Ribot, J. L. and Uppuluri, P. (2019) 'Candida albicans biofilm growth and dispersal: contributions to pathogenesis', *Current opinion in microbiology,* 52, pp. 1–6.

Whaley, S. G. (2018) *Novel Determinants That Influence Azole Susceptibility in Candida glabrata and Candida albicans.* The University of Tennessee Health Science Center.

Zafar, S., Arshad, M. F., Khan, H., Menahil, R., Iqbal, L., Prabhavathi, S. J., Kumar, M. S., Omar, A. F. and Shaheen, T. (2024) 'Nanoformulations of plant essential oils for managing mycotoxins producing fungi: An overview', *Biocatalysis and Agricultural Biotechnology,* 60, pp. 103314.

Zhang, L., Yu, S.-Y., Chen, S. C.-A., Xiao, M., Kong, F., Wang, H., Ning, Y.-T., Lu, M.-Y., Sun, T.-S. and Hou, X. (2020) 'Molecular characterization of Candida parapsilosis by microsatellite typing and emergence of clonal antifungal drug resistant strains in a multicenter surveillance in China', *Frontiers in Microbiology,* 11, pp. 1320.

APPENDICES

Appendix A: Biomedical Research Ethics Committee Approval (UKZN)

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

12 September 2023

Miss Caitlin Ramnarain (217003421)
School of Laboratory Medicine & Medical Science
Medical School

Dear Miss Ramnarain,

Protocol reference number: BREC/00005995/2023
Project title: The effect of yoghurt and lactobacillus isolated from yoghurt on Candida isolates obtained from a cohort of South African women living with and without HIV
Degree: PhD

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 12 September 2023. Please ensure that any outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is valid for one year from 12 September 2023. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on RIG on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2020) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 10 October 2023.

Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

Appendix B: Biomedical Research Ethics Committee Approval (UKZN) – Amendments

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

03 June 2025

Miss Caitlin Ramnarain (217003421)
School of Laboratory Medicine & Medical Science
Medical School

Dear Miss Ramnarain,

Protocol reference number: BREC/00005995/2023
Project title: The effect of yoghurt and lactobacillus isolated from yoghurt on Candida isolates obtained from a cohort of South African women living with and without HIV
Degree: PhD
NEW TITLE: Profiling of vaginal Candida isolates from South African women: Exploring potential alternative therapeutic strategies

I wish to advise you that your application for amendments for change of title, objectives and other amendments received on 27 May 2025 for the above study has been **noted and approved** by a subcommittee of the Biomedical Research Ethics Committee.

The committee will be notified of the above approval at its next meeting to be held on 8 July 2025.

Yours sincerely

[redacted]

.....
Ms A Marimuthu
(for) Prof S Singh
Chair: Biomedical Research Ethics Committee

Appendix C: Published Manuscript from Chapter 2

The Journal of Medical Laboratory Science & Technology South Africa. 2025;7:61-74
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ORIGINAL RESEARCH

Correlation between genotypes and antifungal susceptibility profiles of *Candida* isolates from pregnant and non-pregnant women in South Africa

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Vulvovaginal candidiasis (VVC) is a common vaginal infection, affecting up to 75% of women of reproductive age at some point in their lives. The leading cause of VVC is *Candida albicans* (*C. albicans*). This study investigates the correlation between genotypes and antifungal susceptibility profiles of *Candida* isolates collected from pregnant and non-pregnant South African women. A total of 72 *Candida* isolates were identified using the Applied Biosystems TaqMan assay and confirmed via germ tube tests and polymerase chain reaction (PCR). All isolates (100%) were identified as *C. albicans*. ABC genotyping revealed that 62.5% of isolates were genotype A, 26.4% were genotype B, and 11.1% were genotype C. Antifungal susceptibility testing using the Sensititre™ YeastOne™ YO10 AST Plate assessed minimum inhibitory concentrations (MIC) for anidulafungin, caspofungin, fluconazole, micafungin, and voriconazole. Fluconazole showed the highest resistance rate at 13.9%, while 86.1% of isolates remained susceptible. Genotype A predominated among isolates resistant to anidulafungin, fluconazole, micafungin, and voriconazole. All caspofungin-resistant isolates were genotype C. Genotype B exhibited no resistance to any antifungals tested, indicating the lowest virulence among the genotypes. These findings suggest that genotypes A and C have higher resistance profiles, emphasising the need for routine VVC screening and resistance surveillance to inform effective *Candida* infection management.

Keywords: vulvovaginal candidiasis, drug resistance, polymerase chain reaction, antifungal agents, pregnancy complications

Introduction

VVC is a prevalent vaginal infection, impacting up to 75% of women of reproductive age at least once during their lifetime.[1,2] The primary organism behind VVC is *C. albicans*, which is responsible for 70–90% of VVC cases.[3] Previous research highlighted a high prevalence of *Candida* infections in Africa and the Middle East, with reported rates of 55.18% and 76.92%, respectively.[4] In sub-Saharan and central Africa, the infection rate for *C. albicans* was found to be 22.74%, while the South African region had a slightly lower rate of 22.44%. Candidiasis ranks as the fifth most common life-threatening fungal infection, with an estimated mortality rate of 40%.[5] Symptoms of VVC often include redness around the genital area, inflammation of the genital tract, itching, and a thick, white discharge.[6]

Identifying *Candida* species from culture-positive women is crucial in determining the species responsible for the infection, as well as assessing antimicrobial susceptibility and resistance mechanisms.[7] Accurate *Candida* species identification is important, as their responses to antifungal drugs can differ, which helps ensure effective therapy and reduces the risk of treatment failure. Various molecular techniques, such as Southern blotting, hybridisation, multilocus sequence typing, and deoxyribonucleic acid (DNA) microsatellite analysis, have been employed for genotyping *Candida* isolates. These genotyping methods classify strains into clades, which are groups derived from a common ancestor.

Studies have shown that the distribution of these clades is influenced by geographical factors and antifungal resistance patterns, with certain clades linked to specific resistance profiles.[8] The ABC genotyping method is commonly used for *C. albicans*, where PCR amplification of the 25S ribosomal deoxyribonucleic acid (rDNA) allows classification of the isolates into genotypes A (450 bp [base pair]), B (840 bp), C (450 bp and 840 bp), and D (1 080 bp).[7] A study by Jafarian et al.[9] reported genotype A at a prevalence of 57.9%, genotype B at 31.6%, and genotype C at 10.5% among 933 patients, of whom 23 had confirmed *Candida* infections.

Antifungal drug resistance is a significant contributor to the treatment failure of *Candida* infections.[10] Treatment options for candidiasis remain limited, even though various antifungal drugs exist. Based on their mechanisms of action, antifungal drugs used to manage candidiasis are divided into four classes: (1) disruption of cell membrane sterol (polyenes, such as amphotericin B and nystatin), (2) inhibition of the ergosterol biosynthesis pathway (azoles, including fluconazole, voriconazole, posaconazole, and ravuconazole), (3) inhibition of DNA or ribonucleic acid synthesis (flucytosine), and (4) inhibition

of glucan synthesis (echinocandins, such as caspofungin, micafungin, and anidulafungin).[11]

Fluconazole remains the most frequently used azole for both preventing and treating *Candida* infections. However, prolonged use of this drug can lead to the development of resistance among *Candida* species, reducing its effectiveness. This is supported by previous research, which has shown that resistance to fluconazole among *Candida* species is becoming a growing concern within healthcare systems.[12]

Although global research has advanced in linking *C. albicans* genotypes with antifungal susceptibility profiles, there is a limited amount of international data and a notable lack of data from South Africa. Therefore, addressing this gap is crucial, particularly given the high prevalence of candidiasis in the region and the potential public health implications of increasing treatment resistance. This study aims to explore the correlation between genotypes and the antifungal susceptibility profiles of anidulafungin, caspofungin, fluconazole, micafungin, and voriconazole among *C. albicans* isolates from pregnant and non-pregnant South African women, providing a novel insight into the dynamics of drug resistance. By linking the genotypes to antifungal susceptibility profiles, this research contributes to a better understanding of resistance mechanisms, potentially leading to more effective therapeutic strategies and enhanced regional treatment guidelines.

Methodology

Study setting and population derived from the parent study

This was a sub-study of a broader research project, which focused on diagnosing vaginitis and vaginosis pathogens in women. In the parent study, 150 women were recruited from Victoria Mxenge Hospital in Durban, KwaZulu-Natal, South Africa. Participants in the main study were 18 years or older, provided written informed consent, and agreed to self-collect vaginal swabs, following sample collection instructions from the research team. Data on sexual behaviour, clinical history, and sociodemographic details were gathered from each participant through a structured questionnaire administered by the study team. The recruitment period for the study population spanned from January to August 2022.

Ethical approval for the sub-study

Approval for this sub-study was granted by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (reference number BREC/00005995/2023).

Laboratory procedures

Sample collection and processing of vaginal swabs from the parent study

Following sample collection, the swabs for *Candida* detection were placed in a 15 ml tube with Cary–Blair transport medium (Neogen, United States) and transported to the laboratory for culture analysis. At the laboratory, the swabs were streaked onto Sabouraud dextrose agar (SDA) plates containing chloramphenicol (Neogen, United States) and incubated at 35 °C for 48 hours. After incubation, a total of 72 isolates showed positive *Candida* cultures, with 31 isolates derived from pregnant women and 41 from non-pregnant women. These cultures were stored at -80 °C for future use.

Retrieval from storage for the sub-study

The 72 stored *Candida* cultures were retrieved from storage and sub-cultured onto SDA plates containing chloramphenicol and incubated at 35 °C for 48 hours.

Confirmatory assays for the isolates

The germ tube test

The germ tube test was conducted to distinguish *C. albicans* from other *Candida* species, such as *C. krusei*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*. For this test, 0.5 ml foetal calf serum (Thermo Fisher Scientific, United States) was added to microfuge tubes, and a single colony from the SDA plate culture was mixed into the serum. The tubes were incubated at 37 °C for 2–3 hours. After incubation, wet mount microscopy was used to observe germ tube formation. A positive result for *C. albicans* was indicated by short hyphal (filamentous) extensions emerging laterally from yeast cells without constriction at their origin. Samples without hyphal extensions or with constricted short hyphae at their origin were categorised as negative or as other yeast species.[13]

DNA extraction

DNA extraction from *Candida* cultures was carried out using the PureLink™ Microbiome Kit (Thermo Fisher Scientific, United States) following the manufacturer's protocol. The extracted DNA was stored at -20 °C. A NanoDrop Spectrophotometer (Thermo Fisher Scientific, United States) was used to measure the concentration and purity of the DNA.

Confirmation of Candida isolates by real-time PCR

The identity of *Candida* isolates was verified using the Applied Biosystems TaqMan® Assay (Thermo Fisher Scientific, United States), with commercially available primers and probes targeting *C. albicans*, *C. lusitaniae*, *C. dubliniensis*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, and *C. krusei*. The assays were conducted on the QuantStudio™ 5 Real-Time PCR detection system (Thermo Fisher Scientific, United States). Each PCR reaction had a final volume of 5 µl, consisting of 0.25 µl FAM-labelled probe/primer mix, 1.25 µl FastStart 4X Probe Master Mix (part no. 4444434, Thermo Fisher Scientific, United States), 2 µl template DNA, and 1.5 µl nuclease-free water.

A positive control (TaqMan™ Vaginal Microbiota Extraction Control, cat no. A32039) and a non-template control were included. Amplification involved an initial step at 95 °C for 30 seconds, followed by 45 cycles of denaturation at 95 °C for three seconds, and annealing at 60 °C for 30 seconds. Fluorescent signals from amplified products were detected at the end of the annealing phase, and the QuantStudio™ 5 system software automatically generated raw fluorescent data, including cycle threshold (C_t) mean values.

Genotyping of the C. albicans isolates

The isolates were typed using the ABC genotyping method. The *25S rDNA* gene was amplified from the previously extracted DNA using primers CA–INT–L (5'-ATA AGG GAA GTC GGC AAA ATA GAT CCG TAA-3') and CA–INT–R (5'-CCT TGG CTG TGG TTT CGC TAG ATA GTA GAT-3'). The PCR master mix included 200 nm (nanometres) of each primer, 12.5 µl DreamTaq 2X Master Mix (Thermo Fisher Scientific, United States), 9.5 µl nuclease-free water, and 2 µl template DNA.

The PCR tubes were then placed in a Bio-Rad thermal cycler, and cycling conditions were set to 95 °C for two minutes, followed by 35 cycles of 95 °C for 30 seconds, 60 °C for one minute, and 72 °C for one minute, with a final extension at 72 °C for seven minutes. PCR products were electrophoresed on a 1% agarose gel and visualised using an ultraviolet transilluminator. Based on the yielded band sizes, the *C. albicans* isolates were classified as genotype A (450 bp), genotype B (840 bp), genotype C (450 bp and 840 bp), and genotype D (1 080 bp).[14]

Antifungal susceptibility assay for C. albicans

Susceptibility testing was conducted using the Sensititre™ YeastOne™ YO10 AST Plate (Thermo Fisher Scientific, United States) to assess the MICs of *C. albicans* isolates against anidulafungin, caspofungin, fluconazole, micafungin, and voriconazole. An inoculum of *C. albicans* for each isolate was prepared to match a 0.5 McFarland standard. From this suspension, 20 µl was added to the Sensititre™ YeastOne™ Broth, followed by adding 100 µl of the inoculum into the microtiter plates. The plates were sealed and incubated at 35 °C for 24–25 hours. The *C. albicans* ATCC 10231 strain served as a control strain, while untreated cultures of each isolate were included as growth controls. All experiments were conducted in triplicate. Table I displays the MIC breakpoints, as per the Clinical and Laboratory Standards Institute guidelines.

Table I: MIC breakpoints per the Clinical and Laboratory Standards Institute guidelines

Antifungal	Susceptible (µg/ml)	SDD (µg/ml)	Resistant (µg/ml)
Anidulafungin	≤ 0.25	0.5	≥ 1
Caspofungin	≤ 0.25	0.5	≥ 1
Fluconazole	≤ 2	4	≥ 8
Micafungin	≤ 0.25	0.5	≥ 1
Voriconazole	≤ 0.12	0.25–0.5	≥ 1

MIC – minimum inhibitory concentration, SDD – susceptible-dose dependent

Results

Confirmatory assays for the obtained isolates

All isolates (72/72, 100%) were confirmed as *C. albicans* based on the germ tube test (Figure 1). Further confirmation was achieved through the quantitative PCR assay using primers and probes specific to *Candida* species. Table II presents the amplification results from the TaqMan assay using these specific probes and primers, indicating that all samples yielded positive amplification. The positive and negative controls also produced the expected results.

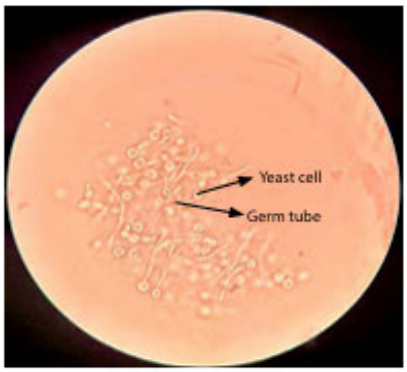

Figure 1: A microscope slide illustrating the results of the germ tube test, which was examined using oil immersion at 100X magnification.

Table II: Results from the TaqMan assay utilising primers and probes specific to *Candida* species, indicating the amplification outcomes for the tested samples

Isolate name	TaqMan assay result (C_t value)
ZMO1	Positive (15.9)
ZMO10	Positive (30.8)
ZMO11	Positive (17.5)
ZMO12	Positive (16.7)
ZMO14	Positive (19.8)
ZMO17	Positive (20.2)
ZMO18	Positive (26.3)
ZMO20	Positive (32.9)
ZMO21	Positive (14.0)
ZMO23	Positive (12.3)
ZMO25	Positive (15.3)
ZMO27	Positive (14.5)
ZMO28	Positive (29.1)
ZMO29	Positive (30.9)
ZMO30	Positive (15.3)
ZMO32	Positive (28.6)
ZMO34	Positive (17.8)
ZMO35	Positive (14.9)
ZMO37	Positive (19.6)
ZMO40	Positive (33.1)
ZMO41	Positive (17)
ZMO42	Positive (31.3)
ZMO43	Positive (17.7)
ZMO44	Positive (24.9)
ZMO47	Positive (16.2)
ZMO53	Positive (28.6)
ZMO54	Positive (17.4)
ZMO56	Positive (17.8)
ZMO58	Positive (17.9)
ZMO59	Positive (18.9)
ZMO60	Positive (15.8)
ZMO62	Positive (24.7)

Table II: Continued

Isolate name	TaqMan assay result (C_t value)
ZMO63	Positive (16.8)
ZMO65	Positive (26.6)
ZMO67	Positive (19.3)
ZMO68	Positive (29.6)
ZMO69	Positive (25.1)
ZMO71	Positive (12.4)
ZMO72	Positive (21.5)
ZMO75	Positive (18.5)
ZMO77	Positive (17)
ZMO79	Positive (27.9)
ZMO80	Positive (19.2)
ZMO81	Positive (22)
ZMO82	Positive (29.0)
ZMO83	Positive (16.5)
ZMO84	Positive (20.2)
ZMO85	Positive (19.1)
ZMO86	Positive (15.4)
ZMO87	Positive (18.8)
ZMO88	Positive (16.9)
ZMO89	Positive (16.4)
ZMO91	Positive (14.6)
ZMO94	Positive (14.9)
ZMO95	Positive (18.8)
ZMO96	Positive (15.1)
ZMO97	Positive (16.3)
ZMO98	Positive (17.9)
ZMO99	Positive (28.3)
ZMO102	Positive (16.0)
ZMO103	Positive (15.2)
ZMO107	Positive (27.2)
ZMO110	Positive (31.1)
ZMO119	Positive (18.8)
ZMO128	Positive (15.9)
ZMO132	Positive (15.5)
ZMO135	Positive (32.2)
ZMO141	Positive (14.9)
ZMO142	Positive (31.1)
ZMO145	Positive (31.7)
ZMO146	Positive (13.6)
ZMO147	Positive (13.7)

C_t – cycle threshold

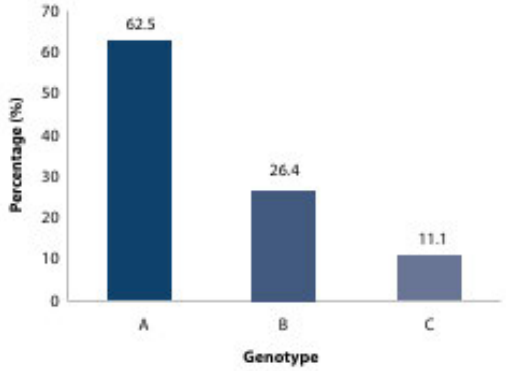

Figure 2: The percentages of *C. albicans* genotypes.

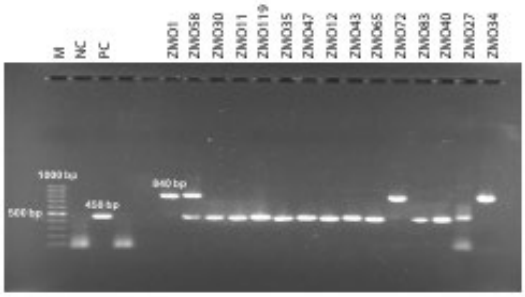

Figure 3: An agarose gel displaying positive amplicons generated for *C. albicans* isolates is shown, with observed band sizes of 450 bp, 840 bp, and a combination of both 450 bp and 840 bp. M represents the 100 bp DNA molecular ladder (ThermoFisher Scientific), NC indicates the negative control (no template DNA added), PC denotes the positive control (*C. albicans* ATCC 10231 strain), along with 15 clinical isolates of *C. albicans*.

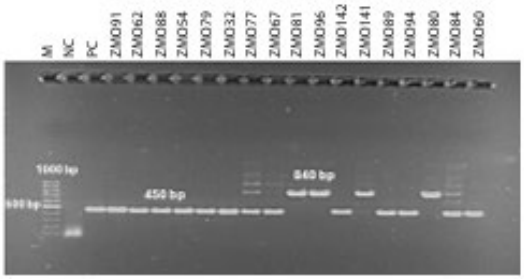

Figure 4: An agarose gel illustrating positive amplicons generated for *C. albicans* isolates is presented, with observed band sizes of 450 bp and 840 bp. M indicates the 100 bp DNA molecular ladder (ThermoFisher Scientific), NC represents the negative control (no template DNA added), PC denotes the positive control (*C. albicans* ATCC 10231 strain), and the gel includes 17 clinical isolates of *C. albicans*.

Genotyping analysis

All 72 isolates (100%) produced positive PCR results (Figures 3, 4, 5, 6, 7, and 8). Figure 2 shows that most of the isolates (45/72, 62.5%) exhibited a 450 bp band, classified as genotype A. Nineteen isolates (19/72, 26.4%) displayed a 840 bp band size, assigned to genotype B. Additionally, eight isolates (8/72, 11.1%) yielded two band sizes of 450 bp and 840 bp, corresponding to genotype C. No isolates were classified as genotype D, since the 1 080 bp band was not detected. A detailed summary of the assigned genotypes is shown in Table III.

Antifungal susceptibility assays

For anidulafungin, 91.7% of the 72 isolates tested (66/72) were susceptible, exhibiting MICs ≤ 0.25 µg/ml. Additionally, 1.4% of

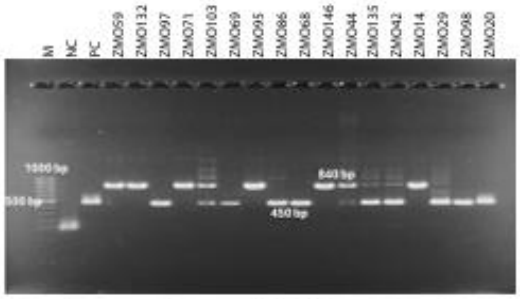

Figure 5: An agarose gel is presented, displaying positive amplicons generated for *C. albicans* isolates. The observed band sizes include 450 bp, 840 bp, and a combination of both 450 bp and 840 bp bands. M indicates the 100 bp DNA molecular ladder (ThermoFisher Scientific), NC represents the negative control (no template DNA added), PC denotes the positive control (*C. albicans* ATCC 10231 strain), along with 17 clinical isolates of *C. albicans*.

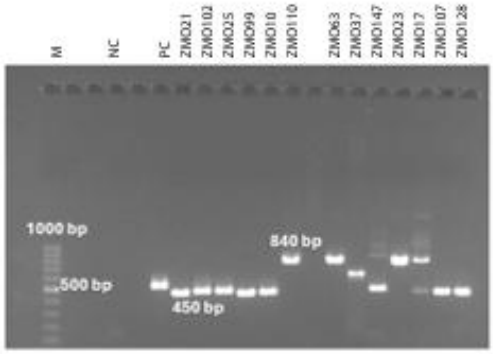

Figure 6: An agarose gel is shown, displaying positive amplicons generated for *C. albicans* isolates. The observed band sizes include 450 bp, 840 bp, and a combination of both 450 bp and 840 bp bands. M denotes the 100 bp DNA molecular ladder (ThermoFisher Scientific), NC indicates the negative control (no template DNA added), PC represents the positive control (*C. albicans* ATCC 10231 strain), and the gel includes 13 clinical isolates of *C. albicans*.

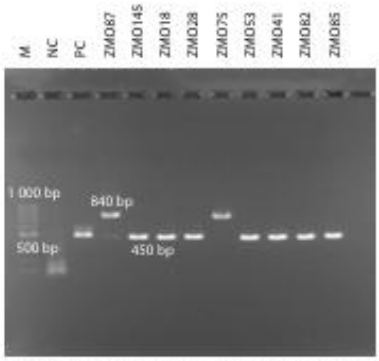

Figure 7: An agarose gel is presented, showing positive amplicons generated for *C. albicans* isolates. The observed band sizes include 450 bp, 840 bp, and a combination of both 450 bp and 840 bp bands. M indicates the 100 bp DNA molecular ladder (ThermoFisher Scientific), NC represents the negative control (no template DNA added), PC denotes the positive control (*C. albicans* ATCC 10231 strain), and the gel contains nine clinical isolates of *C. albicans*.

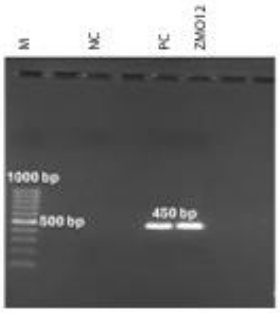

Figure 8: One clinical isolate of *C. albicans* in an agarose gel showing the positive amplicons generated, with observed band sizes at 450 bp

M – 100 bp DNA molecular ladder (Thermo Fisher Scientific), NC – negative control (no template DNA added), PC – positive control (*C. albicans* ATCC 10231 strain)

Table III: Assignment of genotypes for individual isolates based on the banding patterns obtained

Isolate name	PCR product size (bp)	Genotype
ZMO1	840	B
ZMO10	450	A
ZMO11	450	A
ZMO12	450	A
ZMO14	840	B
ZMO17	450 & 840	C
ZMO18	450	A
ZMO20	450	A
ZMO21	450	A
ZMO23	840	B
ZMO25	450	A
ZMO27	450	A
ZMO28	450	A
ZMO29	450	A
ZMO30	450	A
ZMO32	450	A
ZMO34	840	B
ZMO35	450	A
ZMO37	840	B
ZMO40	450	A
ZMO41	450	A
ZMO42	450 & 840	C
ZMO43	450	A
ZMO44	450 & 840	C
ZMO47	450	A
ZMO53	450	A
ZMO54	450	A
ZMO56	450	A
ZMO58	450 & 840	C
ZMO59	840	B
ZMO60	450	A
ZMO62	450	A
ZMO63	840	B

Table III: Continued

Isolate name	PCR product size (bp)	Genotype
ZMO65	450	A
ZMO67	450	A
ZMO68	450	A
ZMO69	450	A
ZMO71	840	B
ZMO72	840	B
ZMO75	840	B
ZMO77	450 & 840	C
ZMO79	450	A
ZMO80	840	B
ZMO81	840	B
ZMO82	450	A
ZMO83	450	A
ZMO84	450 & 840	C
ZMO85	450	A
ZMO86	450	A
ZMO87	840	B
ZMO88	450	A
ZMO89	450	A
ZMO91	450	A
ZMO94	450	A
ZMO95	840	B
ZMO96	840	B
ZMO97	450	A
ZMO98	450	A
ZMO99	450	A
ZMO102	450	A
ZMO103	450 & 840	C
ZMO107	450	A
ZMO110	840	B
ZMO119	450	A
ZMO128	450	A
ZMO132	840	B
ZMO135	450 & 840	C
ZMO141	840	B
ZMO142	450	A
ZMO145	450	A
ZMO146	840	B
ZMO147	450	A

PCR – polymerase chain reaction, bp – base pair

the isolates (1/72) were classified as susceptible-dose dependent (SDD) to anidulafungin, with a 0.5 µg/ml MIC. Meanwhile, 6.9% of the isolates (5/72) showed resistance to anidulafungin, displaying MICs ≥ 1 µg/ml (Supplementary Table I).

For caspofungin, 94.4% of the 72 isolates tested (68/72) were susceptible, exhibiting MICs ≤ 0.25 µg/ml. Additionally, 2.8% of the isolates (2/72) were classified as SDD to caspofungin, with a 0.5 µg/ml MIC. Similarly, 2.8% of the isolates (2/72) were resistant to caspofungin, displaying MICs ≥ 1 µg/ml (Supplementary Table II).

For fluconazole, 86.1% of the 72 isolates tested (62/72) were susceptible, exhibiting MICs ≤ 2 µg/ml. Conversely, 13.9% of the isolates (10/72) were found to be resistant to fluconazole, with MICs ≥ 8 µg/ml (Supplementary Table III).

For micafungin, 90.3% of the 72 isolates tested (65/72) were susceptible, exhibiting MICs ≤ 0.25 µg/ml. Additionally, 5.5% of the isolates (4/72) were classified as SDD to micafungin, with a 0.5 µg/ml MIC. Furthermore, 4.2% of the isolates (3/72) were resistant to micafungin, displaying MICs ≥ 1 µg/ml (Supplementary Table IV).

For voriconazole, 86% of the 72 isolates tested (62/72) were susceptible, exhibiting MICs ≤ 0.12 µg/ml. Additionally, 7% of the isolates (5/72) were classified as SDD to voriconazole, with MICs ranging from 0.25 µg/ml to 0.5 µg/ml. Furthermore, another 7% of the isolates (5/72) were resistant to voriconazole, displaying MICs ≥ 1 µg/ml (Supplementary Table V).

Table IV presents the susceptibility profiles of *C. albicans* isolates against anidulafungin, caspofungin, fluconazole, micafungin, and voriconazole.

Table IV: Susceptibility profiles of *C. albicans* isolates to anidulafungin, caspofungin, fluconazole, micafungin, and voriconazole ($n = 72$)

Antifungal	Susceptibility profile		
	Susceptible	SDD	Resistant
Anidulafungin	66 (91.7%)	1 (1.4%)	5 (6.9%)
Caspofungin	68 (94.4%)	2 (2.8%)	2 (2.8%)
Fluconazole	62 (86.1%)	0 (0%)	10 (13.9%)
Micafungin	65 (90.3%)	4 (5.5%)	3 (4.2%)
Voriconazole	62 (86%)	5 (7%)	5 (7%)

SDD – susceptible-dose dependent

Correlation between susceptibility profiles and genotypes

Table V presents the correlation between anidulafungin susceptibility patterns and *C. albicans* genotypes. Among the 66 isolates that were susceptible to anidulafungin, 41/66 (62.1%) were classified as genotype A, 18/66 (27.3%) as genotype B, and 7/66 (10.6%) as genotype C. The single isolate (1/1, 100%) that was SDD to anidulafungin was assigned to genotype B. Among the five isolates resistant to anidulafungin, 4/5 (80%) were classified as genotype A and 1/5 (20%) as genotype C.

Table VI illustrates the correlation between caspofungin susceptibility patterns and *C. albicans* genotypes. Among the 68 isolates that were susceptible to caspofungin, 43/68 (63.2%) were classified as genotype A, 19/68 (28%) as genotype B, and 6/68 (8.8%) as genotype C. The isolates that were SDD to caspofungin (2/2, 100%) were both assigned to genotype A. Additionally, the isolates resistant to caspofungin (2/2, 100%) were both classified as genotype C.

Table V: Correlation between the susceptibility profile of anidulafungin and *C. albicans* genotypes

Genotype	Susceptibility pattern		
	Susceptible (*n* = 66)	SDD (*n* = 1)	Resistant (*n* = 5)
A	41 (62.1%)	0 (0%)	4 (80%)
B	18 (27.3%)	1 (100%)	0 (0%)
C	7 (10.6%)	0 (0%)	1 (20%)

SDD – susceptible-dose dependent

Table VI: Correlation between the susceptibility profile of caspofungin and *C. albicans* genotypes

Genotype	Susceptibility pattern		
	Susceptible (*n* = 68)	SDD (*n* = 2)	Resistant (*n* = 2)
A	43 (63.2%)	2 (100%)	0 (0%)
B	19 (28%)	0 (0%)	0 (0%)
C	6 (8.8%)	0 (0%)	2 (100%)

SDD – susceptible-dose dependent

Table VII: Correlation between the susceptibility profile of fluconazole and *C. albicans* genotypes

Genotype	Susceptibility pattern	
	Susceptible (*n* = 62)	Resistant (*n* = 10)
A	37 (59.7%)	8 (80%)
B	19 (30.6%)	0 (0%)
C	6 (9.7%)	2 (20%)

SDD – susceptible-dose dependent

Table VIII: Correlation between the susceptibility profile of micafungin and *C. albicans* genotypes

Genotype	Susceptibility pattern		
	Susceptible (*n* = 65)	SDD (*n* = 4)	Resistant (*n* = 3)
A	40 (61.5%)	3 (75%)	2 (66.7%)
B	18 (27.7%)	1 (25%)	0 (0%)
C	7 (10.8%)	0 (0%)	1 (33.3%)

SDD – susceptible-dose dependent

Table IX: Correlation between the susceptibility profile of voriconazole and *C. albicans* genotypes

Genotype	Susceptibility pattern		
	Susceptible (*n* = 62)	SDD (*n* = 5)	Resistant (*n* = 5)
A	37 (59.7%)	5 (100%)	3 (60%)
B	19 (30.6%)	0 (0%)	0 (0%)
C	6 (9.7%)	0 (0%)	2 (40%)

SDD – susceptible-dose dependent

Table VII presents the correlation between fluconazole susceptibility patterns and *C. albicans* genotypes. Among the 62 isolates that were susceptible to fluconazole, 37/62 (59.7%) were classified as genotype A, 19/62 (30.6%) as genotype B, and 6/62 (9.7%) as genotype C. Of the 10 isolates that were resistant to fluconazole, 8/10 (80%) were assigned to genotype A, while 2/10 (20%) were assigned to genotype C.

Table VIII illustrates the correlation between micafungin susceptibility patterns and *C. albicans* genotypes. Among the 65 isolates that were susceptible to micafungin, 40/65 (61.5%) were classified as genotype A, 18/65 (27.7%) as genotype B, and 7/65 (10.8%) as genotype C. Of the four isolates that were SDD to micafungin, 3/4 (75%) were assigned to genotype A, while 1/4 (25%) was assigned to genotype B. Among the three isolates that were resistant to micafungin, 2/3 (66.7%) were classified as genotype A, and 1/3 (33.3%) as genotype C.

Table IX presents the correlation between voriconazole susceptibility patterns and *C. albicans* genotypes. Among the 62 isolates that were susceptible to voriconazole, 37/62 (59.7%) were classified as genotype A, 19/62 (30.6%) as genotype B, and 6/62 (9.7%) as genotype C. All isolates (5/5, 100%) that were SDD to voriconazole were assigned to genotype A. Among the five isolates that were resistant to voriconazole, 3/5 (60%) were classified as genotype A, while 2/5 (40%) were classified as genotype C.

Discussion

Globally, *Candida* species account for a substantial proportion of fungal infections related to women's healthcare.[2] *C. albicans* is responsible for over 80% of yeast infections.[15] This study aimed to link the distribution of *C. albicans* genotypes with the antifungal susceptibility profiles of anidulafungin, caspofungin, fluconazole, micafungin, and voriconazole in 72 *C. albicans* isolates obtained from the vaginal swabs of pregnant and non-pregnant patients at the Victoria Mxenge Hospital in Durban, South Africa.

ABC genotyping analysis of *25S rDNA* is a molecular typing technique widely used to detect variations among *Candida* species, playing an important role in linking specific genotypes to antifungal resistance and serving as a valuable tool for epidemiological research.[7,16] In the current study, most of the 72 isolates were identified as genotype A (62.5%), followed by genotype B (26.4%) and genotype C (11.1%). These findings align with those of another study, which reported genotype A as the most prevalent among all *C. albicans* isolates (54.69%), followed by genotype B (34.38%) and genotype C (10.94%).[17]

This study is consistent with findings from research conducted in Iraq on patients with VVC. Among the 54 *C. albicans* isolates examined in that study, genotype A was the most prevalent, accounting for 50%, followed by genotype B at 29.62%, and genotype C at 20.37%.[18] A study conducted in northeast Brazil on women with vaginal *Candida* infections also identified genotype A as the most prevalent (93.6%), followed by genotype C (6.4%). Notably, genotypes B and D were not detected in that study.[19] However, in a study from Palestine, 55% of the vaginal *Candida* isolates had genotype C, followed by genotypes A (32.4%) and B (12.6%).[20] An Iranian study also found genotype C as the most common (83.5%), followed by genotype B (12.6%) and genotype A (3.9%).[21] These observed genotype variations may be attributed to differences in geographical regions.

The resistance of *Candida* species to antifungal treatments remains a major challenge in managing fungal infections.[22] In this study, we assessed the in vitro susceptibility of 72 *C. albicans* isolates to five antifungal agents: anidulafungin, caspofungin, fluconazole, micafungin, and voriconazole. Anidulafungin, caspofungin, and micafungin are part of the echinocandin class of antifungals, while fluconazole and voriconazole are classified as azole antifungals. Resistance to these drugs can lead to treatment failure. *Candida* species show the highest prevalence of resistance to azole antifungals.

Vaginal *C. albicans* isolates are known to exhibit high resistance rates to fluconazole.[23] Among these isolates, 91.7% demonstrated susceptibility to anidulafungin, while 6.9% exhibited resistance. For caspofungin, 94.4% were susceptible, with 2.8% showing resistance. Regarding fluconazole, 86.1% of isolates were susceptible, and 13.9% were resistant. Micafungin showed a susceptibility rate of 90.3%, with 4.2% resistance. Lastly, voriconazole had a susceptibility rate of 86%, and 7% of isolates were resistant. Caspofungin and micafungin demonstrated lower resistance rates than anidulafungin, fluconazole, and voriconazole. Among the antifungal agents tested, fluconazole exhibited the highest resistance rates.

These results align with findings from another study, which reported resistance rates for caspofungin (1%), fluconazole (9%), and voriconazole (6%).[24] Similarly, a study conducted in China demonstrated low resistance rates against anidulafungin, caspofungin, and micafungin, while higher resistance rates were noted for fluconazole and voriconazole.[25] High resistance rates to fluconazole and voriconazole have also been observed in additional studies from China and Bulgaria.[26,27] In contrast, research conducted in Tanzania revealed lower resistance rates, with 3.1% for fluconazole and 3.6% for voriconazole.[28] Research on antifungal resistance among *Candida* species is valuable as it offers up-to-date data on resistance patterns, aiding in evaluating empirical treatment guidelines. Variations in antifungal susceptibility profiles among *C. albicans* can be attributed to geographic differences and variations in the populations studied.

The current study also described the correlation between the detected *C. albicans* genotypes in relation to the susceptibility profiles of anidulafungin, caspofungin, fluconazole, micafungin, and voriconazole. Of the 66 isolates susceptible to anidulafungin, 62.1% belonged to genotype A, 27.3% were genotype B, and 10.6% were genotype C. Among the five isolates resistant to anidulafungin, 80% were categorised as genotype A, with the remaining 20% classified as genotype C. For caspofungin susceptibility, 63.2% of the 68 isolates were identified as genotype A, 28% as genotype B, and 8.8% as genotype C, while both caspofungin-resistant isolates were genotype C. Of the 62 fluconazole-susceptible isolates, 59.7% were genotype A, 30.6% were genotype B, and 9.7% were genotype C. For the 10 fluconazole-resistant isolates, 80% were identified as genotype A, and 20% as genotype C. Regarding micafungin, 61.5% of the 65 susceptible isolates were genotype A, 27.7% were genotype B, and 10.8% were genotype C. Among the three micafungin-resistant isolates, 66.7% were genotype A, and 33.3% were genotype C. Finally, of the 62 isolates susceptible to voriconazole, 59.7% were genotype A, 30.6% were genotype B, and 9.7% were genotype C. Among the five voriconazole-resistant isolates, 60% were genotype A, and 40% were genotype C.

The study demonstrated that most isolates susceptible to the five antifungal agents were predominantly of genotype A, followed by genotype B, and then genotype C. Among the isolates resistant to anidulafungin, fluconazole, micafungin, and voriconazole, most were identified as genotype A, with the remainder being genotype C. Notably, all isolates resistant to caspofungin were of genotype C. No resistant isolates were classified as genotype B, demonstrating that this genotype had a 0% resistance rate, the lowest among the genotypes studied.

However, other studies conducted in China assessing *C. albicans* genotypes against azole antifungals revealed that genotype A isolates showed lower resistance rates than those of genotype B.[23,29] Another study conducted in Jordan found that all *Candida* isolates collected from women with vaginal candidiasis were susceptible to fluconazole, resulting in a 0% resistance rate among genotypes A, B, and C.[30] Lastly, a study conducted in China revealed that *C. albicans 25S rDNA* genotypes belonging to group A exhibited significantly lower susceptibility rates to fluconazole than genotypes B and C.[31]

To our knowledge, no South African studies have linked the genotypes of *C. albicans* isolates from pregnant and non-pregnant women to the antifungal susceptibility profiles of anidulafungin, caspofungin, fluconazole, micafungin, and voriconazole. In this study, most *C. albicans* isolates were identified as genotype A and exhibited resistance to anidulafungin, fluconazole, micafungin, and voriconazole, while caspofungin-resistant isolates were all classified as genotype C. This indicates that genotypes A and C are more virulent than genotype B. Currently, there is limited research on the correlation between *Candida* genotypes and antifungal susceptibility patterns; a gap in the literature that this study addresses.

Study limitations

The study faced a few limitations. The small sample size may have been a reason for the study's inability to detect non-*albicans Candida* species. Additionally, since the research was conducted in a single geographical area and the participants were all recruited from a single clinic, it may not fully represent the broader population. However, given that Victoria Mxenge Hospital serves as a central tertiary hospital, it does reflect a wider portion of Durban's population.

Conclusion

Most *C. albicans* isolates collected from both pregnant and non-pregnant South African women demonstrated susceptibility to anidulafungin, caspofungin, fluconazole, micafungin, and voriconazole. Genotype A was the most prevalent *C. albicans* genotype among women in Durban, South Africa. The isolates susceptible to the five antifungal agents were mainly genotype A, followed by genotype B, and then genotype C. Among isolates

showing resistance to anidulafungin, fluconazole, micafungin, and voriconazole, the majority were classified as genotype A, with the remaining resistant isolates identified as genotype C. All isolates resistant to caspofungin belonged to genotype C. No resistance to any tested antifungal drugs was found among isolates of genotype B, suggesting it is the least virulent strain of *C. albicans*. Additional research is necessary to investigate the occurrence of other genotypes, such as genotype D, and to establish their correlation with antifungal resistance patterns. Future studies can now focus on the mechanisms behind resistance in local isolates. Currently, antifungal resistance patterns for commonly used treatments of *Candida* infections are not being monitored in our local setting, highlighting a need for resistance surveillance to mitigate the risk of future untreatable infections.

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Conflict of interest

The authors declare no conflict of interest.

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Ethical approval

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (reference number BREC/00005995/2023).

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References

1. Rudramurthy SM, Singh S. *Candida* infections in immunocompetent hosts: pathogenesis and diagnosis. Curr Fungal Infect Rep. 2020;14:233-45. https://doi.org/10.1007/s12281-020-00392-5.
2. Sobel JD. Recurrent vulvovaginal candidiasis. Am J Obstet Gynecol. 2016;214(1):15-21. https://doi.org/10.1016/j.ajog.2015.06.067.
3. Sardi JCO, Silva DR, Anibal PC, et al. Vulvovaginal candidiasis: epidemiology and risk factors, pathogenesis, resistance, and new therapeutic options. Curr Fungal Infect Rep. 2021;15:32-40. https://doi.org/10.1007/s12281-021-00415-9.
4. Omrani AS, Pecen L, Rajek P, Raghubir N, Zigmond J. Prevalence of invasive and superficial *Candida* infections in Africa and Middle East; a systematic review and meta-analysis. Am Soc Microbiol. 2014;1273.
5. Bongomin F, Gago S, Oladele RO, Denning DW. Global and multi-national prevalence of fungal diseases—estimate precision. J Fungus (Basel). 2017;3(4):57. https://doi.org/10.3390/jof3040057.
6. Sim M, Logan S, Goh LH. Vaginal discharge: evaluation and management in primary care. Singapore Med J. 2020;61(6):297-301. https://doi.org/10.11622/smedj.2020088.
7. Fornari G, Vicente VA, Gomes RR, et al. Susceptibility and molecular characterization of *Candida* species from patients with vulvovaginitis. Braz J Microbiol. 2016;47(2):373-80. https://doi.org/10.1016/j.bjm.2016.01.005.
8. Lyon JP, Moraes KC, Moreira LM, Aimbire F, de Resende MA. *Candida albicans*: genotyping methods and clade related phenotypic characteristics. Braz J Microbiol. 2010;41(4):841-9. https://doi.org/10.1590/S1517-83822010000400003.
9. Jafarian H, Gharaghani M, Seyedian SS, Mahmoudabadi AZ. Genotyping, antifungal susceptibility, enzymatic activity, and phenotypic variation in *Candida albicans* from esophageal candidiasis. J Clin Lab Anal. 2021;35(7):e23826. https://doi.org/10.1002/jcla.23826.
10. Lee Y, Puumala E, Robbins N, Cowen LE. Antifungal drug resistance: molecular mechanisms in *Candida albicans* and beyond. Chem Rev. 2020;121(6):3390-411. https://doi.org/10.1021/acs.chemrev.0c00199.
11. Wiederhold NP. The antifungal arsenal: alternative drugs and future targets. Int J Antimicrob Agents. 2018;51(3):333-9. https://doi.org/10.1016/j.ijantimicag.2017.09.002.
12. Bhattacharya S, Sae-Tia S, Fries BC. *Candidiasis* and mechanisms of antifungal resistance. Antibiotics (Basel). 2020;9(6):312. https://doi.org/10.3390/antibiotics9060312.
13. Dolan CT, Ihrke DM. Further studies of the germ-tube test for *Candida albicans* identification. Am J Clin Pathol. 1971;55(6):733-4. https://doi.org/10.1093/ajcp/55.6.733.
14. Mashaly GE-S, Zeid MS. *Candida albicans* genotyping and relationship of virulence factors with fluconazole tolerance in infected pediatric patients. Infect Drug Resist. 2022;15:2035-43. https://doi.org/10.2147/IDR.S344998.
15. Talapko J, Juzbašić M, Matijević T, et al. *Candida albicans*—the virulence factors and clinical manifestations of infection. J Fungi (Basel). 2021;7(2):79. https://doi.org/10.3390/jof7020079.
16. Tapia CV, Hermosilla G, Fortes P, et al. Genotyping and persistence of *Candida albicans* from pregnant women with vulvovaginal candidiasis. Mycopathologia. 2017;182(3-4):339-47. https://doi.org/10.1007/s11046-016-0095-3.
17. Shekhany KAM. Isolation and genotyping of *Candida albicans* involved in vaginal candidiasis among pregnant women in Sulaymaniyah and Erbil cities. Zanco J Med Sci. 2021;25(1):493-502. https://doi.org/10.15218/zjms.2021.012.
18. Noori AM, Bander K, Hamada T. Genotype comparisons of *Candida albicans* from patients with vulvovaginal candidiasis. Egypt Acad J Biol Sci. 2016;8(1):1-5. https://doi.org/10.21608/eajbsg.2016.16472.
19. de Medeiros MAP, de Melo APV, Gonçalves SS, Milan EP, Chaves GM. Genetic relatedness among vaginal and anal isolates of *Candida albicans* from women with vulvovaginal candidiasis in north-east Brazil. J Med Microbiol. 2014;63(Pt 11):1436-45. https://doi.org/10.1099/jmm.0.076604-0.
20. Ali-Shtayeh M, Jamous RM, Alothman NHA, et al. Genotyping and antifungal susceptibility of *Candida albicans* strains from patients with vulvovaginal and cutaneous candidiasis in Palestine. Afr J Microbiol Res. 2015;9(13):952-9. https://doi.org/10.5897/AJMR2014.7350.
21. Gharaghani M, Shabanzadeh M, Jafarian H, Mahmoudabadi AZ. ABC typing and extracellular enzyme production of *Candida albicans* isolated from *Candida* vulvovaginitis. J Clin Lab Anal. 2022;36(1):e24117. https://doi.org/10.1002/jcla.24117.
22. Richter SS, Galask RP, Messer SA, et al. Antifungal susceptibilities of *Candida* species causing vulvovaginitis and epidemiology of recurrent cases. J Clin Microbiol. 2005;43(5):2155-62. https://doi.org/10.1128/JCM.43.5.2155-2162.2005.
23. Liu XP, Fan SR, Bai FY, Li J, Liao QP. Antifungal susceptibility and genotypes of *Candida albicans* strains from patients with vulvovaginal candidiasis. Mycoses. 2009;52(1):24-8. https://doi.org/10.1111/j.1439-0507.2008.01539.x.
24. Al-Aameri DA, Zghair SA, Al-Nuaimi BN, et al. Evaluation of susceptibility of *Candida* species to six antifungal drugs in Iraqi specimens. J Commun Dis. 2024;56(2):53-61. https://doi.org/10.24321/0019.5138.202432.
25. Yan L, Wang X-D, Seyedmousavi S, et al. Antifungal susceptibility profile of *Candida albicans* isolated from vulvovaginal candidiasis in Xinjiang province of China. Mycopathologia. 2019;184(3):413-22. https://doi.org/10.1007/s11046-018-0305-2.
26. Shi Y, Zhu Y, Fan S, et al. Molecular identification and antifungal susceptibility profile of yeast from vulvovaginal candidiasis. BMC Infect Dis. 2020;20(287). https://doi.org/10.1186/s12879-020-04985-w.
27. Hitkova HY, Georgieva DS, Hristova PM, Sredkova MP. Antifungal susceptibility of *Candida albicans* isolates at a tertiary care hospital in Bulgaria. Jundishapur J Microbiol. 2019;12(7):e92079. https://doi.org/10.5812/jjm.92079.
28. Mushi MF, Bader O, Bii C, Groß U, Mshana SE. Virulence and susceptibility patterns of clinical *Candida* spp. isolates from a tertiary hospital, Tanzania. Med Mycol. 2019;57(5):566-72. https://doi.org/10.1093/mmy/myy107.
29. Ge S-H, Wan Z, Li J, et al. Correlation between azole susceptibilities, genotypes, and *ERG11* mutations in *Candida albicans* isolates associated with vulvovaginal candidiasis in China. Antimicrob Agents Chemother. 2010;54(8):3126-31. https://doi.org/10.1128/AAC.00118-10.
30. Al-Groom RM, Ali RRM, Shaqra QMA. Genotypes analysis and antifungal susceptibility of *Candida albicans* strains isolated from women with vaginal candidiasis in Jordan using PCR targeting 25SrDNA and ALT repeat sequences of the RPS. Pak J Med Sci. 2024;40(8):1619-24. https://doi.org/10.12669/pjms.40.8.9811.
31. Wang M, Cao Y, Xia M, et al. Virulence and antifungal susceptibility of microsatellite genotypes of *Candida albicans* from superficial and deep locations. Yeast. 2019;36(5):363-73. https://doi.org/10.1002/yea.3397.

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ORIGINAL RESEARCH

Correlation between genotypes and antifungal susceptibility profiles of *Candida* isolates from pregnant and non-pregnant women in South Africa

Supplementary Material

Supplementary Table I: *C. albicans* isolates: MICs and susceptibility profiles to anidulafungin

Isolate name	MIC (µg/ml)	Susceptibility profile
ATCC	0.12	Susceptible
ZMO1	0.06	Susceptible
ZMO10	0.12	Susceptible
ZMO11	0.12	Susceptible
ZMO12	< 0.015	Susceptible
ZMO14	0.12	Susceptible
ZMO17	0.12	Susceptible
ZMO18	0.06	Susceptible
ZMO20	0.12	Susceptible
ZMO21	0.03	Susceptible
ZMO23	0.03	Susceptible
ZMO25	0.12	Susceptible
ZMO27	0.25	Susceptible
ZMO28	0.12	Susceptible
ZMO29	0.12	Susceptible
ZMO30	0.12	Susceptible
ZMO32	< 0.015	Susceptible
ZMO34	< 0.015	Susceptible
ZMO35	0.12	Susceptible
ZMO37	0.06	Susceptible
ZMO40	0.06	Susceptible
ZMO41	0.03	Susceptible
ZMO42	< 0.015	Susceptible
ZMO43	0.12	Susceptible
ZMO44	0.12	Susceptible
ZMO47	0.12	Susceptible
ZMO53	< 0.015	Susceptible
ZMO54	0.12	Susceptible
ZMO56	0.12	Susceptible
ZMO58	0.12	Susceptible
ZMO59	0.03	Susceptible
ZMO60	0.03	Susceptible
ZMO62	012	Susceptible
ZMO63	0.03	Susceptible
ZMO65	0.12	Susceptible
ZMO67	0.06	Susceptible
ZMO68	1	Resistant
ZMO69	< 0.015	Susceptible
ZMO71	< 0.015	Susceptible
ZMO72	0.12	Susceptible
ZMO75	0.06	Susceptible
ZMO77	0.03	Susceptible
ZMO79	0.12	Susceptible
ZMO80	0.12	Susceptible
ZMO81	< 0.015	Susceptible
ZMO82	< 0.015	Susceptible
ZMO83	0.12	Susceptible
ZMO84	0.12	Susceptible
ZMO85	2	Resistant
ZMO86	< 0.015	Susceptible
ZMO87	0.5	Susceptible-dose-dependent
ZMO88	0.12	Susceptible
ZMO89	0.12	Susceptible
ZMO91	0.06	Susceptible
ZMO94	0.06	Susceptible
ZMO95	0.12	Susceptible
ZMO96	0.12	Susceptible
ZMO97	0.06	Susceptible
ZMO98	0.06	Susceptible
ZMO99	< 0.015	Susceptible
ZMO102	< 0.015	Susceptible
ZMO103	0.12	Susceptible
ZMO107	< 0.015	Susceptible
ZMO110	< 0.015	Susceptible
ZMO119	< 0.015	Susceptible
ZMO128	0.12	Susceptible
ZMO132	< 0.015	Susceptible
ZMO135	4	Resistant
ZMO141	< 0.015	Susceptible
ZMO142	1	Resistant
ZMO145	1	Resistant
ZMO146	0.06	Susceptible
ZMO147	0.06	Susceptible

MICs - minimum inhibitory concentrations

Supplementary Table II: *C. albicans* isolates: MICs and susceptibility profiles to caspofungin

Isolate name	MIC (µg/ml)	Susceptibility profile
ATCC	0.12	Susceptible
ZMO1	0.06	Susceptible
ZMO10	0.5	Susceptible-dose-dependent
ZMO11	0.25	Susceptible
ZMO12	0.06	Susceptible
ZMO14	0.06	Susceptible
ZMO17	0.06	Susceptible
ZMO18	0.03	Susceptible
ZMO20	0.12	Susceptible
ZMO21	0.12	Susceptible
ZMO23	0.06	Susceptible
ZMO25	0.06	Susceptible
ZMO27	0.12	Susceptible
ZMO28	0.06	Susceptible
ZMO29	0.12	Susceptible
ZMO30	0.12	Susceptible
ZMO32	< 0.008	Susceptible
ZMO34	0.12	Susceptible
ZMO35	0.12	Susceptible
ZMO37	0.03	Susceptible
ZMO40	0.06	Susceptible
ZMO41	0.06	Susceptible
ZMO42	1	Resistant
ZMO43	0.12	Susceptible
ZMO44	0.12	Susceptible
ZMO47	0.12	Susceptible
ZMO53	0.03	Susceptible
ZMO54	0.12	Susceptible
ZMO56	0.06	Susceptible
ZMO58	0.12	Susceptible
ZMO59	0.06	Susceptible
ZMO60	0.06	Susceptible
ZMO62	0.06	Susceptible
ZMO63	0.03	Susceptible
ZMO65	0.06	Susceptible
ZMO67	0.06	Susceptible
ZMO68	0.5	Susceptible-dose-dependent
ZMO69	< 0.008	Susceptible
ZMO71	< 0.008	Susceptible
ZMO72	0.06	Susceptible
ZMO75	0.03	Susceptible
ZMO77	0.12	Susceptible
ZMO79	0.12	Susceptible
ZMO80	0.12	Susceptible
ZMO81	0.06	Susceptible
ZMO82	0.06	Susceptible
ZMO83	0.12	Susceptible
ZMO84	0.06	Susceptible
ZMO85	< 0.008	Susceptible
ZMO86	< 0.008	Susceptible
ZMO87	0.25	Susceptible
ZMO88	0.06	Susceptible
ZMO89	0.12	Susceptible
ZMO91	0.06	Susceptible
ZMO94	0.06	Susceptible
ZMO95	0.12	Susceptible
ZMO96	0.03	Susceptible
ZMO97	0.03	Susceptible
ZMO98	0.06	Susceptible
ZMO99	0.06	Susceptible
ZMO102	0.03	Susceptible
ZMO103	0.06	Susceptible
ZMO107	< 0.008	Susceptible
ZMO110	0.03	Susceptible
ZMO119	< 0.008	Susceptible
ZMO128	0.12	Susceptible
ZMO132	< 0.008	Susceptible
ZMO135	8	Resistant
ZMO141	< 0.008	Susceptible
ZMO142	0.25	Susceptible
ZMO145	0.25	Susceptible
ZMO146	0.12	Susceptible
ZMO147	0.06	Susceptible

MICs - minimum inhibitory concentrations

Supplementary Table III: *C. albicans* isolates: MICs and susceptibility profiles to fluconazole

Isolate name	MIC (µg/ml)	Susceptibility profile
ATCC	2	Susceptible
ZMO1	0.25	Susceptible
ZMO10	64	Resistant
ZMO11	32	Resistant
ZMO12	0.25	Susceptible
ZMO14	0.5	Susceptible
ZMO17	0.5	Susceptible
ZMO18	0.25	Susceptible
ZMO20	0.5	Susceptible
ZMO21	< 0.12	Susceptible
ZMO23	0.25	Susceptible
ZMO25	0.5	Susceptible
ZMO27	0.25	Susceptible
ZMO28	0.25	Susceptible
ZMO29	0.5	Susceptible
ZMO30	0.12	Susceptible
ZMO32	< 0.12	Susceptible
ZMO34	0.12	Susceptible
ZMO35	64	Resistant
ZMO37	0.5	Susceptible

ZMO40	0.25	Susceptible
ZMO41	0.5	Susceptible
ZMO42	< 0.12	Susceptible
ZMO43	0.5	Susceptible
ZMO44	32	Resistant
ZMO47	4	Susceptible
ZMO53	< 0.12	Susceptible
ZMO54	0.5	Susceptible
ZMO56	0.25	Susceptible
ZMO58	0.25	Susceptible
ZMO59	0.5	Susceptible
ZMO60	< 0.12	Susceptible
ZMO62	2	Susceptible
ZMO63	0.25	Susceptible
ZMO65	8	Resistant
ZMO67	0.5	Susceptible
ZMO68	0.5	Susceptible
ZMO69	< 0.12	Susceptible
ZMO71	< 0.12	Susceptible
ZMO72	0.25	Susceptible
ZMO75	0.5	Susceptible
ZMO77	< 0.12	Susceptible
ZMO79	2	Susceptible
ZMO80	0.5	Susceptible
ZMO81	0.5	Susceptible
ZMO82	< 0.12	Susceptible
ZMO83	0.25	Susceptible
ZMO84	0.5	Susceptible
ZMO85	8	Resistant
ZMO86	< 0.12	Susceptible
ZMO87	2	Susceptible
ZMO88	1	Susceptible
ZMO89	0.5	Susceptible
ZMO91	0.25	Susceptible
ZMO94	0.25	Susceptible
ZMO95	0.5	Susceptible
ZMO96	< 0.12	Susceptible
ZMO97	0.5	Susceptible
ZMO98	0.5	Susceptible
ZMO99	< 0.12	Susceptible
ZMO102	< 0.12	Susceptible
ZMO103	2	Susceptible
ZMO107	< 0.12	Susceptible
ZMO110	< 0.12	Susceptible
ZMO119	< 0.12	Susceptible
ZMO128	32	Resistant
ZMO132	< 0.12	Susceptible
ZMO135	> 256	Resistant
ZMO141	< 0.12	Susceptible
ZMO142	8	Resistant
ZMO145	16	Resistant
ZMO146	< 0.12	Susceptible
ZMO147	0.25	Susceptible

MICs - minimum inhibitory concentrations

Supplementary Table IV: *C. albicans* isolates: MICs and susceptibility profiles to micafungin

Isolate name	MIC (µg/ml)	Susceptibility profile
ATCC	0.015	Susceptible
ZMO1	< 0.008	Susceptible
ZMO10	0.25	Susceptible
ZMO11	0.015	Susceptible
ZMO12	0.015	Susceptible
ZMO14	0.015	Susceptible
ZMO17	0.015	Susceptible
ZMO18	0.015	Susceptible
ZMO20	0.015	Susceptible
ZMO21	0.015	Susceptible
ZMO23	0.015	Susceptible
ZMO25	0.015	Susceptible
ZMO27	0.015	Susceptible
ZMO28	< 0.008	Susceptible
ZMO29	0.5	Susceptible-dose-dependent
ZMO30	0.015	Susceptible
ZMO32	< 0.008	Susceptible
ZMO34	0.015	Susceptible
ZMO35	0.015	Susceptible
ZMO37	< 0.008	Susceptible
ZMO40	< 0.008	Susceptible
ZMO41	< 0.008	Susceptible
ZMO42	< 0.008	Susceptible
ZMO43	0.015	Susceptible
ZMO44	0.015	Susceptible
ZMO47	0.015	Susceptible
ZMO53	< 0.008	Susceptible
ZMO54	0.015	Susceptible
ZMO56	0.015	Susceptible
ZMO58	< 0.008	Susceptible
ZMO59	0.015	Susceptible
ZMO60	< 0.008	Susceptible
ZMO62	0.015	Susceptible
ZMO63	< 0.008	Susceptible
ZMO65	0.06	Susceptible
ZMO67	< 0.008	Susceptible
ZMO68	1	Resistant
ZMO69	< 0.008	Susceptible
ZMO71	< 0.008	Susceptible
ZMO72	0.015	Susceptible
ZMO75	< 0.008	Susceptible
ZMO77	0.03	Susceptible

ZMO79	0.06	Susceptible
ZMO80	0.03	Susceptible
ZMO81	< 0.008	Susceptible
ZMO82	< 0.008	Susceptible
ZMO83	0.015	Susceptible
ZMO84	0.03	Susceptible
ZMO85	2	Resistant
ZMO86	< 0.008	Susceptible
ZMO87	0.5	Susceptible-dose-dependent
ZMO88	0.015	Susceptible
ZMO89	0.015	Susceptible
ZMO91	< 0.008	Susceptible
ZMO94	< 0.008	Susceptible
ZMO95	0.03	Susceptible
ZMO96	0.015	Susceptible
ZMO97	0.015	Susceptible
ZMO98	0.015	Susceptible
ZMO99	< 0.008	Susceptible
ZMO102	< 0.008	Susceptible
ZMO103	0.03	Susceptible
ZMO107	< 0.008	Susceptible
ZMO110	< 0.008	Susceptible
ZMO119	< 0.008	Susceptible
ZMO128	0.015	Susceptible
ZMO132	< 0.008	Susceptible
ZMO135	> 8	Resistant
ZMO141	< 0.008	Susceptible
ZMO142	0.5	Susceptible-dose-dependent
ZMO145	0.5	Susceptible-dose-dependent
ZMO146	0.015	Susceptible
ZMO147	< 0.008	Susceptible

MICs - minimum inhibitory concentrations

Supplementary Table V: *C. albicans* isolates: MICs and susceptibility profiles to voriconazole

Isolate name	MIC (µg/ml)	Susceptibility profile
ATCC	0.06	Susceptible
ZMO1	< 0.008	Susceptible
ZMO10	1	Resistant
ZMO11	0.5	Susceptible-dose-dependent
ZMO12	< 0.008	Susceptible
ZMO14	0.015	Susceptible
ZMO17	< 0.008	Susceptible
ZMO18	< 0.008	Susceptible
ZMO20	0.015	Susceptible
ZMO21	< 0.008	Susceptible
ZMO23	< 0.008	Susceptible
ZMO25	< 0.008	Susceptible
ZMO27	< 0.008	Susceptible
ZMO28	< 0.008	Susceptible
ZMO29	< 0.008	Susceptible
ZMO30	< 0.008	Susceptible
ZMO32	< 0.008	Susceptible
ZMO34	< 0.008	Susceptible
ZMO35	2	Resistant
ZMO37	0.015	Susceptible
ZMO40	< 0.008	Susceptible
ZMO41	< 0.008	Susceptible
ZMO42	< 0.008	Susceptible
ZMO43	< 0.008	Susceptible
ZMO44	1	Resistant
ZMO47	0.25	Susceptible-dose-dependent
ZMO53	< 0.008	Susceptible
ZMO54	< 0.008	Susceptible
ZMO56	< 0.008	Susceptible
ZMO58	< 0.008	Susceptible
ZMO59	< 0.008	Susceptible
ZMO60	< 0.008	Susceptible
ZMO62	0.12	Susceptible
ZMO63	< 0.008	Susceptible
ZMO65	0.12	Susceptible
ZMO67	< 0.008	Susceptible
ZMO68	< 0.008	Susceptible
ZMO69	< 0.008	Susceptible
ZMO71	< 0.008	Susceptible
ZMO72	< 0.008	Susceptible
ZMO75	< 0.008	Susceptible
ZMO77	< 0.008	Susceptible
ZMO79	0.25	Susceptible-dose-dependent
ZMO80	< 0.008	Susceptible
ZMO81	< 0.008	Susceptible
ZMO82	< 0.008	Susceptible
ZMO83	< 0.008	Susceptible
ZMO84	0.015	Susceptible
ZMO85	0.12	Susceptible
ZMO86	< 0.008	Susceptible
ZMO87	0.03	Susceptible
ZMO88	0.06	Susceptible
ZMO89	< 0.008	Susceptible
ZMO91	< 0.008	Susceptible
ZMO94	< 0.008	Susceptible
ZMO95	< 0.008	Susceptible
ZMO96	< 0.008	Susceptible
ZMO97	< 0.008	Susceptible
ZMO98	< 0.008	Susceptible
ZMO99	< 0.008	Susceptible
ZMO102	< 0.008	Susceptible
ZMO103	0.12	Susceptible
ZMO107	< 0.008	Susceptible
ZMO110	< 0.008	Susceptible

ZMO119	< 0.008	Susceptible
ZMO128	1	Resistant
ZMO132	< 0.008	Susceptible
ZMO135	> 8	Resistant
ZMO141	< 0.008	Susceptible
ZMO142	0.5	Susceptible-dose-dependent
ZMO145	0.5	Susceptible-dose-dependent
ZMO146	< 0.008	Susceptible
ZMO147	< 0.008	Susceptible

MICs - minimum inhibitory concentrations